

A MORPHOMETRIC EXAMINATION OF THE ULTRASTRUCTURE
OF REGENERATING HYPOGLOSSAL MOTONEURONS FROM THE
RAT AND ANALYSIS OF THYROID HORMONE INFLUENCE ON
MOTONEURON STRUCTURE AND REGENERATION

1988

HALL

Report Documentation Page				Form Approved OMB No. 0704-0188	
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1. REPORT DATE JUL 1988		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE A Morphometric Examination of the Ultrastructure of Regenerating Hypoglossal Motoneurons from the Rat and Analysis of Thyroid Hormone Influence on Motoneuron Structure and Regeneration				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Uniformed Services University Of The Health Sciences Bethesda, MD 20814				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 227	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			



UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
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Title of Thesis: A Morphometric Examination of the
Ultrastructure of Regenerating Hypoglossal
Motoneurons from the Rat and Analysis of
Thyroid Hormone Influence on Motoneuron
Structure and Regeneration

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ABSTRACT

Title of Dissertation: A MORPHOMETRIC EXAMINATION OF THE ULTRASTRUCTURE OF REGENERATING HYPOGLOSSAL MOTONEURONS FROM THE RAT AND ANALYSIS OF THYROID HORMONE INFLUENCE ON MOTONEURON STRUCTURE AND REGENERATION

Laura Lee Hall, Doctor of Philosophy, 1988

Dissertation directed by: Keith R. Latham, Ph.D.
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The ultrastructure of regenerating hypoglossal motoneurons and their response to altered thyroid hormone status were examined. Morphometric techniques were utilized to evaluate uninjured and axotomized hypoglossal motoneurons from the rat. Hypoglossal nucleus volume and neuronal size were estimated from 50 micron sections. The volume and surface area of cell body organelles were determined from one micron and ultrathin sections. Seven to 30 days following axotomy the volume of the hypoglossal nucleus was significantly diminished ($p < 0.05$). Significant neuronal enlargement ($p < 0.05$) was detected 13 and 21 days following axotomy. Subcellular alterations were most prominent 7 days following nerve transection: nucleolar volume was augmented, rough endoplasmic reticulum surface area was reduced, and non-Golgi smooth membrane surface area increased ($p < 0.05$). Other organelles resisted the influence of axotomy. Ultrastructural parameters returned to control levels by 30 days following the nerve transection.

The influence of thyroid hormone status on the

retrograde response to axotomy was examined. Among hyperthyroid animals, hypoglossal nucleus volume resisted post-axotomy reduction ($p < 0.05$) until 30 days following axotomy. Axotomy-induced neuronal enlargement was prolonged among the hyperthyroid animals. Enhanced nucleolar vacuolization, multiple nucleoli and nuclear filaments were sometimes observed among axotomized motoneurons from hyperthyroid animals. Among axotomized hypoglossal motoneurons from hypothyroid animals, neuronal enlargement was absent. Nuclear filaments were occasionally observed within injured motoneurons from hypothyroid animals. Nuclear eccentricity and deformation were dramatically exacerbated among the regenerating neurons from hypothyroid rats.

The influence of thyroid hormone status on axonal regeneration was examined by (1) evaluating the retrograde axonal transport of horseradish peroxidase (HRP) and (2) measuring tongue force production evoked by nerve stimulation. Excessive circulating thyroid hormone did not appear to influence the rate of axonal regeneration since HRP was first detected in the axotomized hypoglossal nuclei of eu- and hyperthyroid rats 18 days following nerve transection; functional motor recovery was apparent 21 days following axotomy. Among hypothyroid rats, neurons labeled with HRP were initially detected 21 days following axonal transection. Functional reinnervation was first observed 30 days post-axotomy within the hypothyroid group. Thus,

experimentally-induced hypothyroidism resulted in a diminished rate of hypoglossal nerve regeneration and functional recovery. These data suggest that thyroid hormone status does influence regenerating motoneurons.

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ANALYSIS OF THYROID HORMONE INFLUENCE ON MOTONEURON
STRUCTURE AND REGENERATION

by

Laura Lee Hall

Dissertation submitted to the Faculty of the Department of
Physiology Graduate Program of the Uniformed Services
University of the Health Sciences in partial
fulfillment of the requirements for the degree of
Doctor of Philosophy 1988

DEDICATION

To Matthew Pazaryna, my beloved husband
and
Francis and Betty Hall, my dear dad and mom.

ACKNOWLEDGEMENTS

I give special thanks to Dr. Rosemary C. Borke, whose guidance, support, generosity and friendship have been invaluable to me. She introduced me to the fascinating world of neuroscience. Her dedication and pursuit of excellence in all facets of her work are standards by which I will guide my own career.

I am grateful to Dr. Prabir K. Chakraborty, who has been consistently supportive of me throughout my graduate studies. His belief in my ability and generous advice have been a source of encouragement.

I thank Dr. Jack McKenzie for his assistance on my committee and his dedication to graduate education. I am grateful to Dr. Keith R. Latham for his support of my dissertation work. Also, I thank Drs. Tony Lo and Howard Bryant for their contribution to my committee.

I wish to thank Marty Nau and Darryl Harris, who taught me electron microscopy. Their expertise and friendship are appreciated. I acknowledge the generous editorial assistance of Mr. Chuck Sass.

I extend my warmest appreciation to Drs. Connie Pechura and Gary Bennett for their support and friendship throughout this, sometimes difficult, journey. I greatly admire their openness, integrity and approach to science.

I extend a special thanks to my parents, Francis and Betty Hall. From the beginning, they have showered me with love, have encouraged me to pursue my dreams and have had unwavering faith in me.

Finally, I am forever grateful to my husband, Matthew. He has never ceased to believe in me and patiently has endured my sometimes mad pursuit of this goal. His devotion is the wellspring of my happiness.

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RATIONALE

Many previous studies have indicated that the neuronal cell body suffers structural changes following axonal transection (Lieberman, 1971). This retrograde reaction may reflect regenerative activity within the neuron. However, the specific ultrastructural responses of the neuronal perikarya to axotomy have been poorly defined, especially among regenerating mammalian neurons. In the current studies, it was hypothesized that the volume and surface area of organelles within the neuronal cell body may be significantly altered by axotomy. This hypothesis was experimentally addressed by adapting morphometric techniques to the study of the ultrastructure of uninjured and regenerating motoneuron cell bodies.

Thyroid hormone status has been shown to influence peripheral nerve regeneration (Cockett & Kiernan, 1973; McIsaac & Kiernan, 1975; Berenberg et al., 1977; Talman, 1979). Since the neuronal cell body is the protein synthetic support center for the axon, it was hypothesized that altered thyroid hormone status may alter neuronal cell body structure. In a second group of experiments, the influence of thyroid hormone status upon the structure of the neuronal cell body was investigated. Additionally, the rate of hypoglossal nerve regeneration was assessed among the eu-, hyper- and hypothyroid animals.

INTRODUCTION

The Appearance of Axotomized Neurons

The injured neuron has been the subject of experimental scrutiny for nearly 100 years. The irreversible and devastating clinical symptoms often produced by trauma to the nervous system have provoked much of this interest in the neuron and its regenerative ability. The response of the neuronal cell body to axonal lesion has been of particular interest, since the cell body is the synthetic support center for the axon. Axonal lesion can provoke profound morphologic and metabolic changes within the neuronal cell body. This axon or retrograde reaction, (Lieberman, 1971) was first described by Franz Nissl in the late nineteenth century (Nissl, 1892). Nissl observed cell body swelling, peripheral displacement of the nucleus and reduced cytoplasmic basophilia, or chromatolysis, of axotomized facial motoneurons of the rabbit.

Since Nissl's description of the retrograde reaction, many investigators have closely examined the structure of axotomized neurons (Barron, 1983). Some general cytological features have been ascribed to injured neurons. Such generalizations concerning the retrograde reaction, however, belie the extreme variability of the response of the cell body to axotomy (Lieberman, 1974). The type and location of the axon lesion, the population of neurons analyzed and the species and developmental state of the animal influence the retrograde response, confounding

the classical description (Cragg, 1970; Lieberman, 1974). Moreover, various reports have assigned conflicting attributes to a single population of axotomized neurons. The diversity of the retrograde changes which follow axotomy as well as the lack of precise and objective quantitative data concerning the ultrastructure of injured neurons have obscured the relationship of those responses to the regenerative capacity of a neuron (Grafstein, 1983).

Neuronal Cell Body Enlargement Following Axotomy

As originally suggested by Nissl, neuronal cell body enlargement has been qualitatively observed among various axotomized neurons (Lieberman, 1971). In one quantitative analysis of facial motoneurons (FMN) from the hamster, a 25% increase in neuronal cell body cross-sectional area was detected 4 days following axonal transection (LaVelle & LaVelle, 1958). Among goldfish retinal ganglion cell (RGC), a two-fold increase of somal cross-sectional area was measured 13-30 days post-axotomy (Whitnall & Grafstein, 1982, 1983). An increase in neuronal cross-sectional area also has been observed among regenerating hypoglossal motoneurons of the rat (Aldskogius et al., 1980). The appearance of neuronal cell body enlargement among many populations of regenerating neurons and following nerve growth factor (NGF) administration (Hendry and Campbell, 1976) suggests a correlation between neuronal enlargement and anabolic activity.

The Cell Nucleus Response to Axotomy

Eccentric displacement of the cell nucleus and "ruffling" of the nuclear envelope were also included in the earliest description of the retrograde reaction (Nissl, 1892). Many reports have described nuclear enlargement, eccentric displacement of the nucleus and invagination of the nuclear envelope among injured neurons (Lieberman, 1971). Measurements of nuclear cross-sectional area have failed to corroborate the occurrence of nuclear enlargement among axotomized hypoglossal motoneurons (Aldskogius et al., 1980). Several studies have quantitatively analyzed the nuclei of other regenerating neurons. Transection of the facial nerve of the adult hamster led to an early, measured increase of nuclear cross-sectional area but an unchanging nuclear perimeter and boundary density (length of nuclear envelope in relationship to nuclear area) among FMN (LaVelle & LaVelle, 1958; Jones & LaVelle, 1985). RGC of the goldfish, which show a tremendous regenerative capacity, exhibited a reduction of nuclear fractional volume 13 and 30 days following optic tract section (Whitnall & Grafstein, 1982, 1983). The diminished nuclear fractional volume, however, probably reflected cellular enlargement rather than any change in nuclear size. Among spinal motor neurons of the cat, nuclear cross-sectional area was temporarily reduced 2 to 4 weeks following axotomy (Barron et al., 1982).

The cell nucleus reaction to axotomy appears diverse

among different neuronal populations, obscuring the significance of this structural response. Also, limited quantitative data concerning surface area curtails the discussion of this response. Alterations of nuclear shape and position among developing neurons (LaVelle & LaVelle, 1958; Borke, 1983) and neurons following NGF administration (Levi-Montalcini et al., 1968), similar to those observed among axotomized neurons, imply a correlation between cell nucleus structure and the functional state of the cell.

The Nucleolar Response to Axonal Transection

Nucleolar enlargement and vacuolization have often been observed among regenerating neurons (Lieberman, 1971). Among FMN of the hamster and RGC of the goldfish nucleolar fractional volume and cross-sectional area increased following axotomy (LaVelle & LaVelle, 1958; Murray & Grafstein, 1969; Whitnall & Grafstein, 1982, 1983). In the goldfish, nucleolar enlargement and proliferation temporally corresponded to augmented tritiated-proline labeling of the nucleus and nucleolus indicating increased ribonucleoprotein synthesis (Whitnall & Grafstein, 1982, 1983). Among injured FMN of the hamster, the intranucleolar body, which is composed of tightly packed granules of ribonucleoproteins, was depleted (Kinderman & LaVelle, 1976). Injured FMN also expressed more vacuolization following axotomy, which has been correlated with an increase in nucleolar metabolism (Goessens, 1984). Nucleolar enlargement and enhanced

nucleolar metabolism have also been reported for regenerating hypoglossal motoneurons (Watson, 1965; Aldskogius et al., 1980). Additionally, using a careful quantitative approach, Rees demonstrated significant nucleolar eccentricity in the motoneurons following hypoglossal nerve transection (Rees, 1971). The morphologic and metabolic nucleolar responses to axotomy appear to be uniformly expressed by these regenerating populations of neurons. In contrast to the above reports, regenerating spinal motor neurons of the kitten and the cat failed to demonstrate either nucleolar enlargement or heightened nucleolar metabolism following axotomy (Cova & Barron, 1981; Barron et al., 1982).

Chromatolysis

The hallmark of the retrograde reaction is chromatolysis, yet changes in the disposition of the rough endoplasmic reticulum (RER) are not universal and may be the most difficult to qualitatively assess. Generally, following axotomy, the long, flat cisterns of RER are replaced by abbreviated structures and the parallel arrays are disrupted (Lieberman, 1971). While this disorganization has been attributed to a dissolution or loss of the large quantity of RER (Lieberman, 1971), changes in RER surface area are unknown. To date, only one study quantitatively analyzed the RER within regenerating neurons. Among the RGC of the goldfish the volume fraction of RER per cell

diminished 6 days following axonal lesion; thirteen and 30 days following the axotomy, the RER fractional volume had recovered (Whitnall & Grafstein, 1982, 1983).

The Response of Mitochondria, Lysosomes and Golgi complexes to Axotomy

The effects of axotomy upon other organelles in the perikaryon of neurons are poorly documented (Lieberman, 1971). Mitochondrial enlargement, hypertrophy and hyperplasia within axotomized neurons have been suggested by qualitative analysis (Lieberman, 1971). Among regenerating hypoglossal motoneurons, qualitative observations have failed to distinguish any alteration of mitochondrial size (Kirkpatrick, 1968; Borke, 1983). Also, mitochondrial fractional volume was constant among uninjured and 6 days post-axotomy hypoglossal motoneurons (Kirkpatrick; 1968). In the RGC of the goldfish, the fractional volume of mitochondria was unchanged 6 days following axotomy (Whitnall & Grafstein, 1983). An apparent increase in lysosomal number and/or size has been described among axotomized neurons (Lieberman, 1971), and the qualitative impression of lysosomal proliferation was observed among regenerating hypoglossal motoneurons (Kirkpatrick, 1968; Borke, 1983). In the only regenerating system in which lysosomal fractional volume was quantified at several times following axotomy, no such increase was observed (Whitnall & Grafstein, 1982, 1983). Peripheral displacement and

fragmentation of the Golgi apparatus following axotomy have been described among chromatolytic neurons, although most qualitative, ultrastructural analyses have not confirmed this observation (Lieberman, 1971). Among hypoglossal motoneurons following axotomy, one qualitative report described an "increased prominence of the Golgi zone" (Kirkpatrick, 1968), although another qualitative report failed to mention such a response among axotomized hypoglossal motoneurons (Borke, 1983). In the single quantitative analyses of the Golgi apparatus within regenerating neurons, the fractional volume did not significantly change within RGC of the goldfish 6 to 30 days following optic tract lesion (Whitnall & Grafstein, 1982, 1983).

The Significance of the Retrograde Reaction

Broad generalizations about the components of the retrograde reaction are limited by the scope of many of the experiments and conflicting results. Undoubtedly, the retrograde reaction reflects many phenomena, ranging from general post-injury disruption, degeneration and regeneration (Grafstein, 1983). Distinct neuronal populations probably possess a different liability to these events. The structural correlates of these diverse events remain largely unknown, although the similarity of some of the post-injury ultrastructural events and those seen in neurons following NGF administration and during development

suggests the possible anabolic significance of some of the structural configurations.

The structure of the cell body, nucleus and especially cytoplasmic organelles of only a few regenerating neuronal systems have been quantitatively analyzed. The most carefully studied system is the RGC of the goldfish. This neuron may not be an ideal model for mammalian nerve regeneration, since it is located in the central nervous system and exhibits a dramatic regenerative potential uncharacteristic of mammalian central neurons (Grafstein, 1983). Thus, a quantitative analysis of the structure of regenerating neurons from a mammalian system may help identify components of the retrograde reaction important for peripheral nerve regeneration.

Use of Morphometry

The structure of a cell can be quantitatively analyzed by using stereology, a method in which two-dimensional images are used to estimate the three-dimensional components (Weibel, 1973; Williams, 1977). As is apparent from the description of the retrograde reaction, cellular structure is rich in information, however, the precise resolution of structural details by qualitative analysis is limited. Stereological techniques, which are based on geometric probability theory, have evolved as tools to circumvent the problems associated with tissue structure analysis. This approach can expose small or gradual

alterations in structure and thus reveal unknown and unsuspected relationships between structural and functional data. Morphometric or stereological techniques have been used to study many types of tissue, including the nervous system, but there has been little application of this approach to the study of neuronal cell body ultrastructure (Haug, 1986).

Morphometric Evaluation of Regenerating Hypoglossal Motoneurons

In this investigation, the tools of morphometry have been utilized to analyze regenerating hypoglossal motoneurons. While the ultrastructure of injured hypoglossal neurons has been characterized qualitatively (Kirkpatrick, 1968; Fernando, 1973; Borke, 1983) and their physiochemical properties have been investigated (Brattgard et al., 1958; Watson, 1965, 1968, 1970; Smith et al., 1984), a detailed morphometric evaluation of the ultrastructural response has not been reported for this population. In addition, no previous study has quantitatively examined the cell body response of a regenerating mammalian neuron. A primary goal of this study was to quantitatively examine the cell body response of regenerating hypoglossal motoneurons.

Thyroid Hormone Status and Nerve Regeneration

Because nerve injury poses such a serious, clinical problem, research has been directed toward identifying

trophic substances which can facilitate neuronal regeneration. Thyroid hormones have been reported to have a neurotrophic effect following axotomy. Most of the research involving thyroid hormone status and injured neurons has focused upon the recovery of nerve function and axonal elongation. Twenty years ago, the influence of thyroxine on the recovery of injured nervous tissue was first analyzed in the spinal cord (Harvey & Srebnik, 1967). Following compression of the spinal cord, locomotion of the hindlimbs was subjectively assessed in thyroxine-treated and control rats. A greater recovery among the hormone-treated animals was reported. In similar studies however, only a minimal stimulatory effect of thyroxine upon the injured spinal cord was detected (Tator & van Der Jagt, 1980; Tator et al., 1983).

The influence of thyroid hormones upon regenerating, peripheral motor nerves has also been evaluated. Administration of excessive triiodothyronine (T3) accelerated sciatic nerve regeneration in the rat as detected by histological assessment of the nerve and neuromuscular junction (Cockett & Kiernan, 1973; McIsaac & Kiernan; 1975a). These structural data were later correlated to accelerated functional recovery of the sciatic nerve following thyroid hormone treatment (McIsaac & Kiernan, 1975b). Others reported mixed results following thyroid hormone treatment and sciatic nerve injury (Berenberg et al., 1977; Cotrufo et al., 1979; Allpress &

Pollock, 1986). T_3 treatment following crush of the facial nerve influenced the morphology of the regenerating axons without promoting functional recovery (Stelmack & Kiernan, 1977).

T_3 administration did not appear to expedite regeneration of the rabbit hypoglossal nerve following a crush injury (Frizell & McLean, 1979), and in the rat, thyroid hormone treatment only minimally influenced hypoglossal nerve regeneration (Yu & Srinivasan, 1981). The influence of hyperthyroidism on neuronal regeneration and a possible mechanism of action are unclear. The likelihood of an interaction between thyroid hormones and injured neurons is apparent due to its effect on axonal structure and protein synthesis in the injured cells of the nodose ganglion (Cook & Kiernan, 1976). Conversely, a recent study has shown that hypothyroidism significantly depressed nerve regeneration (Talman, 1979). Thus, normal levels of circulating thyroid hormone are probably necessary for the regenerative process, but the mechanism of this action is unknown.

Thyroid Hormone Action

Thyroid hormones have many physiological effects. They are known to be important modulators of development, growth and metabolism (Tata, 1974). The molecular mechanism of action of thyroid hormones is less well understood. A great deal of evidence suggests that T_3 interacts

specifically with nuclear binding sites to alter nucleic acid and protein synthesis, although non-nuclear binding sites do exist (Latham et al., 1978; Dillmann, 1985). There is a good correlation between thyroid hormone responsive tissue and nuclear binding activity (Oppenheimer, 1979).

Thyroid hormone action in the mature brain remains obscure since the hormone's best characterized metabolic effects are absent in adult nervous tissue (Eayrs, 1960). High affinity, low capacity binding sites have been measured in the adult brain (Eberhardt et al., 1978; Schwartz & Oppenheimer, 1978). In fact, there appears to be a higher concentration of nuclear T_3 receptors in the adult brain than in the liver (Eberhardt et al., 1978). More recent studies have identified neurons, as opposed to glia, as the predominant source of nuclear binding activity in the adult CNS (Kolodny et al., 1985; Yokota et al., 1986). Using autoradiographic labeling with ^{125}I -triiodothyronine, the presumable distribution of thyroid hormone target cells in the brain was found to be quite distinct (Dratman et al., 1982). Specific neuronal systems appeared to be targets of thyroid hormone action, including motoneurons of the hypoglossal nucleus. These data suggest that thyroid hormone responsive neurons in the adult brain are distributed in a specific, rather than a generalized pattern.

The Influence of Thyroid Hormone Status on Regenerating Hypoglossal Motoneurons

A second major focus of this study was to analyze the influence of thyroid hormone status upon regenerating hypoglossal motoneurons in the rat. The ultrastructure of regenerating hypoglossal motoneurons was analyzed qualitatively and quantitatively among hyper- and hypothyroid animals; these observations were compared with data from euthyroid animals. Additionally, the influence of thyroid hormone status upon the rate of neuronal regeneration was examined. Hypoglossal motoneurons of the rat were selected for this analysis because the qualitative appearance as well as the metabolism of hypoglossal motoneurons following axotomy have been extensively evaluated and autoradiographic evidence has suggested that this population is rich in thyroid hormone receptors (Dratman et al., 1982). Only a few studies have examined the effect of thyroid hormone status on the regeneration of the hypoglossal nerve (Frizell & McLean, 1979; Talman, 1979; Yu & Srinivasan, 1981). The literature suggests a neuronal site of action of thyroid hormones in the adult nervous system. This work reflects the first evaluation of the influence of thyroid hormone status upon the structural aspects of the retrograde response. These experiments, therefore, offer a unique approach to the possible influence of thyroid hormones on injured neurons.

MATERIAL AND METHODS

EXPERIMENTAL DESIGN

Three groups of experiments were performed. In the first study, an extensive morphometric analysis of uninjured and axotomized hypoglossal motoneurons was conducted in adult (euthyroid) rats. Hypoglossal nuclei from unoperated animals ($n = 3$) as well as those contralateral to axotomized hypoglossal nuclei ($n = 12$) constituted the control group. Three hypoglossal nuclei were examined 7, 13, 21 and 30 days following hypoglossal nerve transection, yielding a total of 12 experimental animals. A total of 15 animals (30 hypoglossal nuclei) were examined in this study.

In the second experimental series, the influence of thyroid hormone status upon the response of hypoglossal motoneurons to axotomy was examined. Uninjured (control) hypoglossal nuclei and axotomized hypoglossal nuclei from hyper- and hypothyroid rats were analyzed qualitatively and quantitatively. The morphometric parameters utilized in this study are indicated in table 1. Control hypoglossal nuclei and axotomized hypoglossal nuclei (7, 13, 21 and 30 days following axon transection), from hyper- and hypothyroid animals, were examined as described for the euthyroid group. A total of 15 hyperthyroid animals and 15 hypothyroid animals were included in this experiment.

The final focus of this investigation was to

determine the influence of thyroid hormone status upon the rate of hypoglossal nerve regeneration. Two methods were employed to detect hypoglossal nerve regeneration following axotomy: (1) the retrograde labeling of axotomized hypoglossal motoneurons after horseradish peroxidase (HRP) was injected into the tongue and (2) force production by the tongue in response to hypoglossal nerve stimulation. Hypoglossal nerve regeneration was examined in control, unoperated animals and in experimental animals 13, 18, 21, 30, 40 and 50 days following axotomy. A total of 14 hyperthyroid animals, 14 euthyroid animals and 18 hypothyroid animals were utilized for this study.

EXPERIMENTAL PROCEDURES

Animal Population

Male, Sprague-Dawley rats (Charles Rivers) were used in this study. Animals were housed in central quarters and maintained on a standard ad libitum diet of food pellets and water. They were exposed to a twelve hour light/dark cycle.

Animals, surgically thyroidectomized by Charles Rivers at three weeks of age, composed the hypothyroid group. Hyperthyroidism was induced by daily intraperitoneal injection of l-thyroxine (50 ug/100g body weight; Sigma), beginning 10 days before any experimental procedure. Crystalline thyroxine was dissolved in ethanol and diluted to the final concentration with Ringer's solution. The

general appearance and weight of the animals were monitored regularly.

Hypoglossal Nerve Injury

All animals were 8 to 9 weeks of age at the time of axotomy. Deep anesthesia was induced by intraperitoneal injection of 7% aqueous chloral hydrate (0.5 ml (Sigma)/100g body weight) and supplemented as needed. Under aseptic conditions, the left hypoglossal nerve was exposed under a stereomicroscope. A midline incision was made at the level of the hyoid bone and the salivary glands were reflected to the right side. The left hypoglossal nerve was transected where it crosses the external carotid artery. The two ends of the nerve were opposed but not sutured. The incision was then sutured. After regaining consciousness, the rats were returned to the general animal quarters.

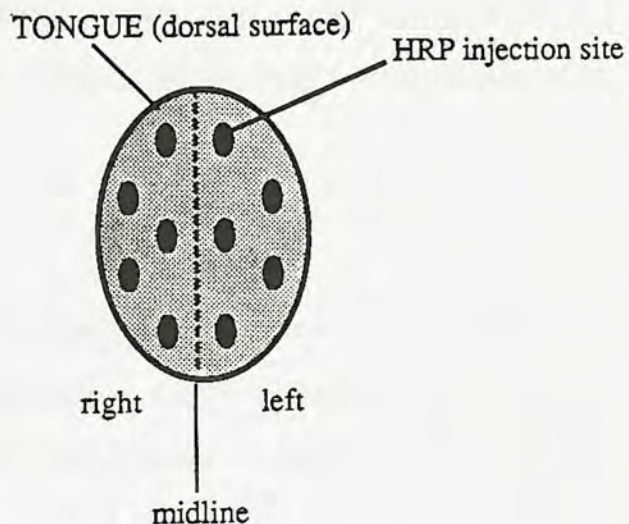
Retrograde Labeling of Hypoglossal Motoneurons with HRP

Under chloral hydrate anesthesia, HRP was injected into the tongue of all rats utilized to demonstrate hypoglossal nerve regeneration. Crystalline HRP (type VI, Sigma) was dissolved in Ringer's solution and frozen in 50 ul aliquots. At both 36 and 24 hours prior to euthanasia, a 30 (hypothyroid) or 50 (eu- and hyperthyroid) ul 10% HRP solution was injected into several sites on both sides of the tongue using a Hamilton syringe (Fig 1). The hyper- and euthyroid rats were administered a total of 100 ul of the

HRP solution (10.0 mg of HRP); hypothyroid animals received a total volume of 60 μ l of the 10% solution (6.0 mg of HRP) commiserate with their smaller body weight.

Figure 1. Diagram of HRP injection procedure

utilized. Among the eu- and hyperthyroid animals, each HRP injection site received 5 μ l of solution. Among the hypothyroid animals, each site received 3 μ l of solution.



Nerve Stimulation

Prior to fixative perfusion, animals used to investigate the function of the hypoglossal nerve were anesthetized with chloral hydrate and prepared for nerve stimulation. The right and left hypoglossal nerves were exposed and loosely ligated. Suture was threaded through the midline of the tongue and attached to a force displacement transducer (Grass, model FT106). The output was directed to a calibrated recorder. The rat's head was firmly secured to prevent measurement artifact due to movement. Bipolar electrodes were applied to the right

(uninjured) hypoglossal nerve and the tongue length yielding a maximal isometric force was determined. This position of the tongue was utilized for subsequent force measurements. Square stimuli of increasing voltages (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 V), 2.5 ms in duration, were applied sequentially to the uninjured and injured hypoglossal nerves. Three stimuli per voltage level were administered at 20 second intervals.

Fixation

The abdominal and thoracic cavities of animals, anesthetized with chloral hydrate, were exposed and blood was collected from the left ventricle with an 18 gauge needle prior to transcardial perfusion. Following blood collection, an 18 gauge, blunt needle, fastened to the end of a perfusion apparatus, was inserted through an incision in the left ventricle and secured with a hemostat in the opening of the ascending aorta. The descending aorta in the abdominal cavity was occluded with a hemostat to maximize fluid delivery to the brain. The right atrium was incised. Ringer's solution (100 ml) was initially introduced, followed by an aldehyde fixative; all solutions were maintained at room temperature. Two different fixative regimens were utilized. Animals that were injected with HRP were perfused with 400-700 ml of phosphate buffered paraformaldehyde (2.5%) - glutaraldehyde (1.25%) solution. All other animals were perfused with a double stage aldehyde

fixative: (1) 200-350 ml of sodium cacodylate buffered paraformaldehyde (1%) - glutaraldehyde (1.25%) solution, followed by (2) sodium cacodylate buffered paraformaldehyde (4%) - glutaraldehyde (5%) solution (Reese & Karnovsky, 1967).

Tissue Preparation

Two to three hours following perfusion, the brain was removed from the cranium and 50 micron coronal sections were cut through the rostro-caudal extent of the medulla on the Vibratome. Alternate sections were mounted on slides. Selected remaining tissue sections were processed for electron microscopy by: (1) washing with 0.1 M sodium cacodylate buffer, (2) post-fixing for one hour in 1% osmium tetroxide (EMS) in the cacodylate buffer at room temperature, (3) washing in the buffer solution, (4) incubating in tannic acid solution (Simionescu & Simionescu, 1976), (5) washing again with the buffer solution, (6) dehydrating in ethanol and xylene and (7) embedding in Epon resin (EMS).

Sections which were reacted for HRP were washed initially in 0.1 M Tris buffer, pH = 7.6, incubated for 10 minutes in 0.5% cobalt chloride and rewashed in the Tris buffer solution. Following several washes with sodium phosphate buffer (0.1 M), the 50 μ m sections were incubated for 90 minutes in fresh diaminobenzidine (DAB; Sigma) solution (DAB, B-D-glucose, NH_4Cl , glucose oxidase,

phosphate buffer) at 37 C (Itoh et al., 1981). The reacted tissue sections were mounted on slides.

All mounted 50 micron sections were stained with cresyl violet and examined using a Nikon BIOPHOT light microscope.

Alternate 50 micron sections were embedded in plastic. One micron sections were cut from randomly selected plastic embedded tissue blocks with glass knives on the Sorvall MT6000 ultramicrotome. The one micron sections were stained with toluidine blue-pyronin-Y and analyzed using a Nikon BIOPHOT light microscope. Ultrathin sections of silver interference were cut from the tissue blocks with a diamond knife (DuPont) on the Sorvall MT6000 microtome. The ultrathin sections were collected on 200 mesh copper grids (EMS), stained with lead citrate for 5 minutes and examined and photographed using a Phillips 401 Transmission Electron Microscope.

Measurement of Total Serum 1-Thyroxine

Blood samples were stored for 30 minutes at 5 C in plain test tubes. Following centrifugation of the sample, the serum was stored at -20 C until it was assayed. Total circulating 1-thyroxine was measured using a solid-phase ^{125}I radioimmunoassay (Diagnostic Products Corporation).

STEREOLOGICAL PROCEDURES

Stereological procedures previously described were adapted to this study (Weibel 1973; Williams, 1977). A list of formulas used to estimate each parameter is given in Table 1.

Sampling Method

A stratified method of sampling the tissue (Fig 2) for morphometric analysis was used in this study (Weibel, 1973). Fifty micron coronal sections were systematically chosen from the medulla for estimation of hypoglossal nucleus volume and neuronal cell body size. Estimates of these parameters were derived from every fourth 50 micron section.

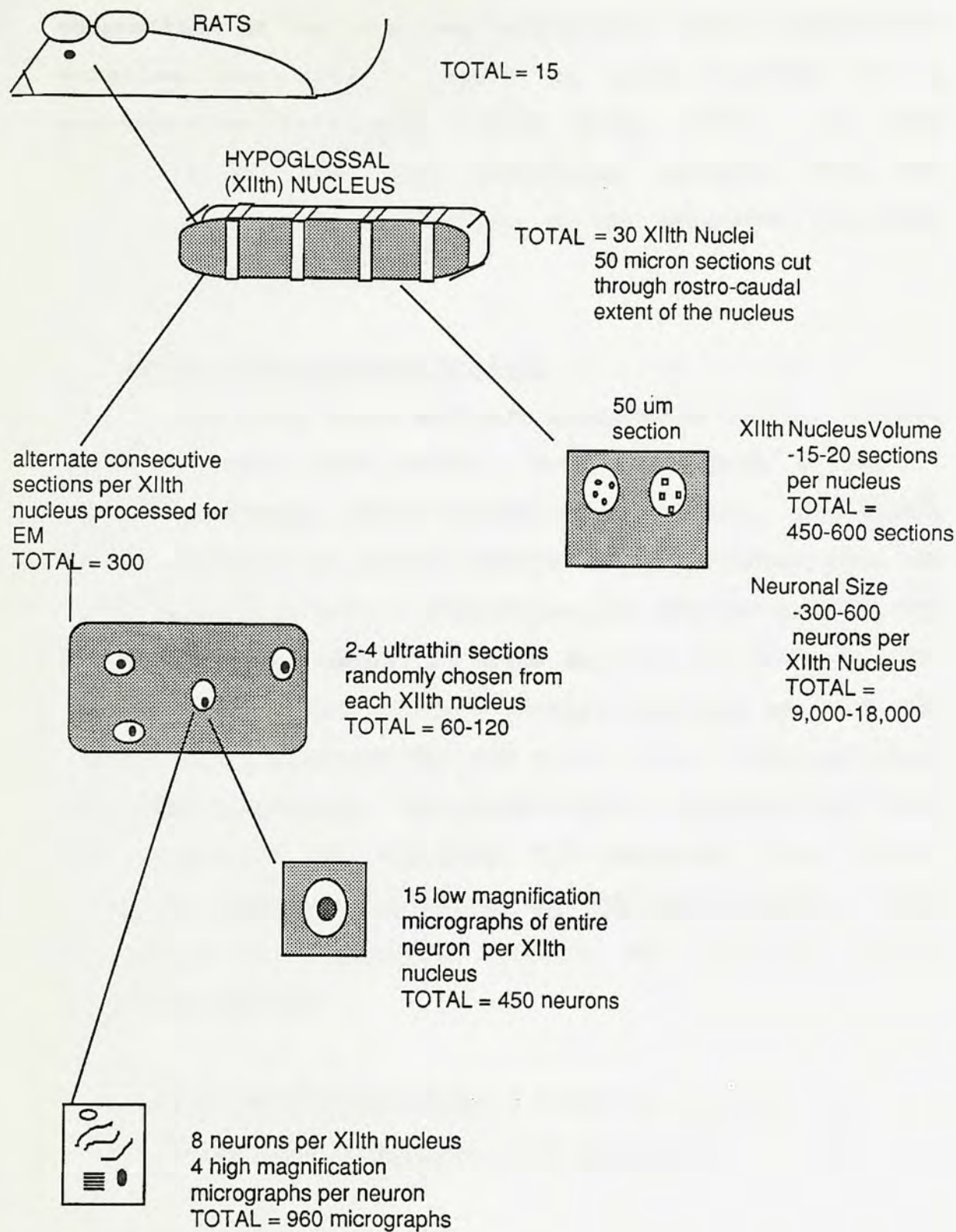
Alternate 50 micron sections through the rostro-caudal extent of the hypoglossal nucleus were processed for electron microscopy. From this group, one micron and ultrathin sections were obtained from 3 randomly selected plastic-embedded tissue sections. The sections were analyzed systematically, beginning in the upper left hand corner of the field viewed in the light and electron microscope. Using this method, neuronal cell bodies containing a nucleus and not obscured by a grid bar were included in the sample until a total of 8 to 15 neurons were obtained from each hypoglossal nucleus. Two to 4 sections were necessary to achieve this sample number per hypoglossal nucleus. To avoid prejudice in selecting the cytoplasmic

Table 1. Estimated Morphometric Parameters

Parameter	Estimator
XIIth ^a Nucleus Volume (mm ³) ^b	[XIIth nucleus xs ^c -area x 0.2 mm] / (magnification) ²
Neuronal Fractional Volume (%) ^b	[neuronal xs-area/ XIIth nucleus xs-area] x 100
Total Neuronal Volume (mm ³) ^b	(neuronal fractional volume) x (XIIth nucleus volume)
Nuclear Fractional Volume (%) ^b	[nuclear xs-area/ neuronal xs-area x 100
Total Nuclear Volume (mm ³) ^b	(nuclear fractional volume) x (total neuronal volume)
Nuclear Surface Density (/cm) ^b	[nuclear perimeter/ neuronal xs-area] x (4/pi) x (magnification)
Nuclear Surface Area (mm ²) ^b	[(nuclear surface density) x (total neuronal volume)]/ 10
Nucleolar Fractional Volume (%) ^b	[nucleolar xs-area/ nuclear xs-area] x 100
Total Nucleolar Volume (μm ³) ^b	[(nucleolar fractional volume) x (total nuclear volume)] x 10 ⁹
Mitochondrial & Lysosomal Fractional Volume (%)	[organelle xs-area/ cytoplasmic xs-area] x 100
Total Organelle Volume (μm ³)	[(organelle fractional volume) x (neuronal total volume - nuclear total volume)] x 10 ⁹
RER, Golgi and Smooth Membrane Surface Density (/cm)	[# intersections with organelle/ line in cytoplasm] x (2) x (magnification)
Organelle Surface Area (mm ²)	[(organelle surface density) x (neuronal total volume - nuclear total volume)]/ 10

^ahypoglossal^bparameters used to evaluate influence of thyroid hormone status upon neuronal structure^ccross-sectional

Figure 2. Flow chart illustrating the stratified sampling technique utilized in this study.



composition at the electron microscopic level, subcellular sampling was done visually by gross criteria at a magnification less than 2,000X (Loud, 1968). At this magnification the fine structural details were not discernible. Four photographs of the cytoplasm (28,080X) per neuron were utilized.

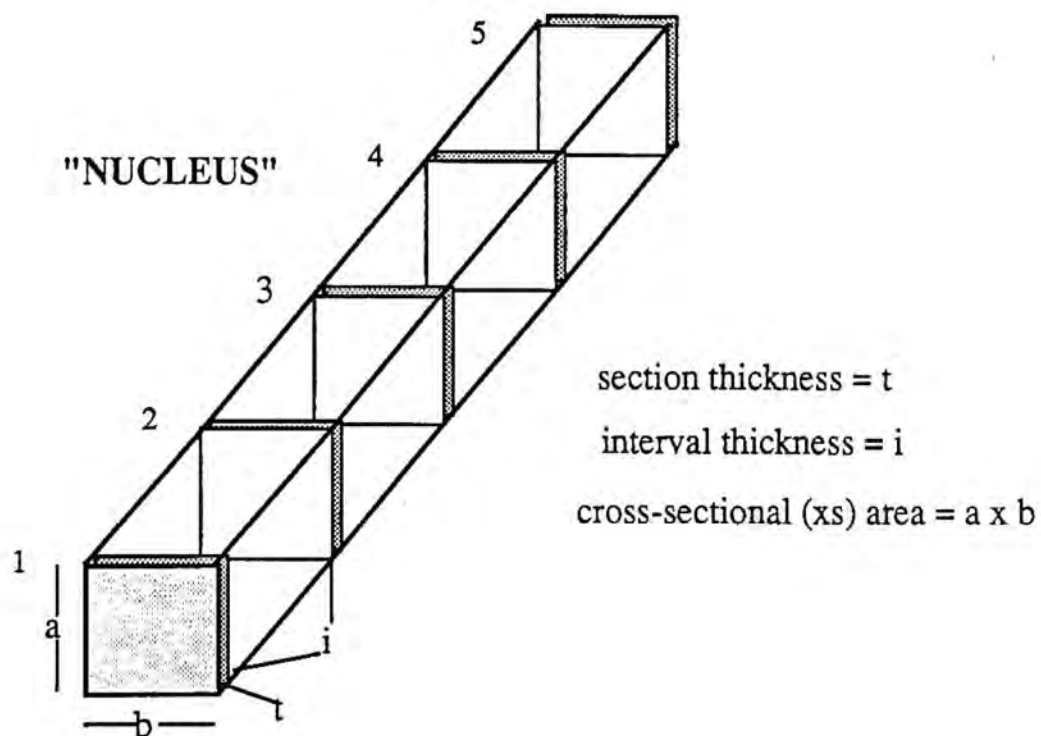
Volume of the Hypoglossal Nucleus

The total right and left hypoglossal nuclear volumes (Fig 3), from each animal, were determined by serial planimetry (Shenk, 1979; Uylings et al., 1986). The images of the selected 50 micron sections were projected from the light microscope onto a digitizing pad (Microplan II). The interval between sample sections equaled 150 microns. The cross-sectional area of each section was measured with the digital planimeter and the sum total of all cross-sectional areas was calculated. The actual total cross-sectional area was determined by dividing the measured total cross-sectional area by the square of the magnification. The volume of the hypoglossal nucleus was estimated by the following formula:

$$\text{Volume} = (\text{section thickness} + \text{interval thickness}) \times (\text{total measured cross-sectional area/magnification}^2) \quad (1)$$

or,

Figure 3. Illustration of method utilized to estimate hypoglossal nucleus volume. Cross-sectional area ($a \times b$) was measured with a digital planimeter.



Volume of "Nucleus" = (xs-area 1 + xs-area 2 + ... + xs-area 5) \times (t + i)

Figure 3

$$\text{Volume} = (50 \text{ um} + 150 \text{ um}) \times$$

$$(\text{total measured cross-sectional area/magnification}^2). \quad (2)$$

Various landmarks were used to identify the boundaries of the hypoglossal nucleus including: (1) medially, the junction of the contralateral hypoglossal nucleus, (2) ventrally, the medial longitudinal fasciculus and emerging fibers of the hypoglossal nerve, (3) laterally, myelinated bundles of fibers in the adjacent reticular formation and (4) dorsally, the dorsal motor nucleus of the vagus in the lower medulla and the floor of the fourth ventricle in the upper medulla. The most caudal and rostral boundaries of the nucleus were designated as sections containing at least four neuronal profiles.

Neuronal Size

Parameters utilized to examine neuronal cell body size included: neuronal fractional volume (V_v), total neuronal volume, average neuronal cross-sectional area and average neuronal maximal diameter. The sample of 50 micron sections used to estimate hypoglossal nucleus volume was utilized. The image of each section was projected from the light microscope onto the digitizing pad and each neuronal profile was traced with the digital planimeter. Neuronal V_v and total neuronal volume were calculated by the formulas:

$$\text{Neuronal } V_V = (\text{total cross-sectional area of neurons} / \text{total cross-sectional area of hypoglossal nucleus}) \quad (3)$$

and,

$$\text{Total Neuronal Volume} = (\text{Neuronal } V_V) \times (\text{Hypoglossal Nucleus Volume}). \quad (4)$$

Cell Nucleus Volume and Surface Area

Using the digital planimeter, the cross-sectional area of the neuronal cell body and cell nucleus as well as the length of the cell nucleus perimeter were measured from low magnification (5,070X - 6,500X) electron photomicrographs of each neuron. The formulas to calculate cell nucleus V_V and total cell nucleus volume were, respectively,:

$$\text{Nucleus } V_V = (\text{total cross-sectional area of the cell nucleus} / \text{total neuronal cross-sectional area}) \quad (5)$$

and,

$$\text{Total Nucleus Volume} = (\text{Nucleus } V_V) \times (\text{Total Neuronal Volume}). \quad (6)$$

The surface density (S_V) of the cell nucleus was calculated for each hypoglossal nucleus using the following formula:

$$\text{Nuclear } S_V = 4/\pi \times \text{magnification} \times (\text{total nuclear perimeter} / \text{total neuronal cross-sectional area}). \quad (7)$$

Total nuclear surface area was calculated by the following formula:

$$\text{Total Nuclear Surface Area} = (\text{Nuclear } S_V) \times (\text{Total Neuronal Volume}). \quad (8)$$

Nucleolar Volume

Neurons containing a nucleus were utilized to determine nucleolar V_V and total nucleolar volume. The cross-sectional area of the cell nucleus and, when present, nucleolus were measured using a digital planimeter. Estimates of nucleolar V_V and total volume were derived from the following formulas:

$$\text{Nucleolar } V_V = (\text{total nucleolar cross-sectional area}) / (\text{total cell nucleus cross-sectional area}), \quad (9)$$

and,

$$\text{Total Nucleolar Volume} = (\text{Nucleolar } V_V) \times (\text{Total Cell Nucleus Volume}). \quad (10)$$

Mitochondrial and Lysosomal Volume

Electron photomicrographs (final magnification =

28,080 X) of the cytoplasm from each neuron were used to determine mitochondrial and lysosomal volume. The fractional volume (V_V) of these organelles was determined from planimetric measurements of their cross-sectional area:

$$\text{Organelle } V_V = (\text{total cross-sectional area of the organelle}) / (\text{total cross-sectional area of the perikaryon}). \quad (11)$$

Total mitochondrial and lysosomal volume were estimated by:

$$\begin{aligned} \text{Total Organelle Volume} = & (\text{Organelle } V_V) \times \\ & (\text{Total Neuronal Cell Body Volume} - \text{Total Cell} \\ & \text{Nucleus Volume}). \end{aligned} \quad (12)$$

Classical descriptions of the organelles provided the basis for positive identification. (Peters et al., 1976)

Surface Area of Cytoplasmic Membranes

The surface density (S_V) and total surface area of the rough endoplasmic reticulum (RER), Golgi apparatus and non-Golgi smooth membrane were estimated. The electron photomicrographs used to measure mitochondrial and lysosomal volume were also utilized to estimate these parameters. Random test lines, drawn on transparencies and generated from a random numbers chart, were superimposed on each photomicrograph. Intersections with the membrane structure and the test lines were counted. Also, the total length of test lines within the neuronal perikaryon were measured.

The formula used to calculate S_V was:

$$\text{Membrane } S_V = (2) \times (\text{magnification}) \times (\text{total number of intersections with organelle/ total length of test lines in the perikaryon}). \quad (13)$$

Total surface area of the membranes was estimated by:

$$\text{Total Membrane Surface Area} = (\text{Membrane } S_V) \times (\text{Total Neuronal Volume} - \text{Total Cell Nucleus Volume}). \quad (14)$$

The organelles were positively identified on the basis of classical descriptions (Peters et al., 1976). Smooth membrane were identified as membranous organelles with no ribosomal studding and not associated with a Golgi complex. Subsurface cisterns were not sampled.

Analysis of Neuronal HRP Labeling

Three to four Vibratome sections, from representative regions of the longitudinal extent of the hypoglossal nucleus, were examined for HRP labeling. In each section, the total number of neurons in the left and right hypoglossal nuclei were counted, as were the number of neurons with peroxidase reaction product. The percent labeling of neurons within each hypoglossal nucleus was determined by:

% HRP Labeling per Hypoglossal Nucleus = (# of HRP positive neurons within a hypoglossal nucleus/ total # of neurons counted within the hypoglossal nucleus) X 100.

(15)

The percent HRP labeling in the injured versus uninjured hypoglossal nucleus was defined as:

% HRP Labeling in Injured/Uninjured Hypoglossal Nucleus = (# of HRP positive neurons within injured hypoglossal nucleus/ # of HRP positive neurons within uninjured contralateral hypoglossal nucleus) X 100.

(16)

STATISTICAL ANALYSIS

Preliminary morphometric data from 4 euthyroid and 2 hypothyroid control animals were analyzed to determine the variability present within each sampling level (ie. variation between animals, sections, neurons and photomicrographs) (Shay, 1975). The calculated standard error of the mean (SEM) was utilized to determine the final animal, cell and photomicrograph number by extrapolating variance estimates with increasing sample number. All calculations were performed by a nested ANOVA program (Snedecor & Cochran, 1980) generously provided by Dr. Robert Buschmann (Veteran's Administration, Chicago, IL).

Means \pm SEM are reported. One-way analysis of variance was utilized to compare the morphometric data from

the hypoglossal nuclei of unoperated control animals and from those contralateral to axotomy (Snedecor & Cochran, 1980). Morphometric data collected from animals at different intervals following axotomy were analyzed by one-way ANOVA. When significant effects were observed, such data were further analyzed by Student-Newman-Keul's test to statistically compare group means (Snedecor & Cochran, 1980). Data involving total neuronal number and HRP-labeled neurons were analyzed using a corrected Chi-square analysis (Snedecor & Cochran, 1980). Maximal force and stimulus threshold data from the injured and uninjured hypoglossal nerve within each animal were analyzed using a paired t-test (Snedecor & Cochran, 1980). Computations were performed with the Systat (Systat, Inc.) statistical program package.

THE RETROGRADE RESPONSE AMONG THE EUTHYROID POPULATIONThe Response of the Hypoglossal Nucleus Contralateral to Axotomy

No qualitative differences were observed among hypoglossal neurons contralateral to axotomy or unoperated controls when examined with light or electron microscopy. Additionally, statistical comparison of data from various control groups (normal, contralateral to transection) failed to reveal any significant ($p > 0.05$) differences. Therefore, such data were combined for further analysis.

Light Microscopic Observations of Vibratome Sections

Neurons, glial cells, blood vessels and neurites were observed in 50 micron sections of uninjured hypoglossal nuclei (Fig 4a). In the perikarya of the motoneurons, basophilic patches of Nissl substance were abundant. Seven to 30 days following axotomy the appearance of the hypoglossal nuclei were altered (Fig 4b). The cross-sectional profile of hypoglossal nuclei appeared diminished in some sections. Also, the number of glial profiles throughout the neuropil and adjacent to neurons seemed to increase. In some sections, the perikarya motoneurons appeared enlarged, especially 13 to 21 days following axotomy. The perikarya of motoneurons were hyperchromatic 7

Figure 4a. In this 50 micron coronal section through the medulla of a euthyroid rat, the cross-sectional area of the left and right uninjured hypoglossal nuclei (outlined) appear similar. Densely stained neurons are present within the nuclei (48X).

Figure 4b. The left (L) hypoglossal nucleus (outlined), pictured in this 50 micron section, suffered axotomy 13 days prior to fixation. The cross-sectional profile of the injured hypoglossal nucleus appears somewhat diminished when compared to the contralateral, uninjured nucleus (R). Also in the injured hypoglossal nucleus, the neurons appear somewhat enlarged, and there is increased glial cells (48X).

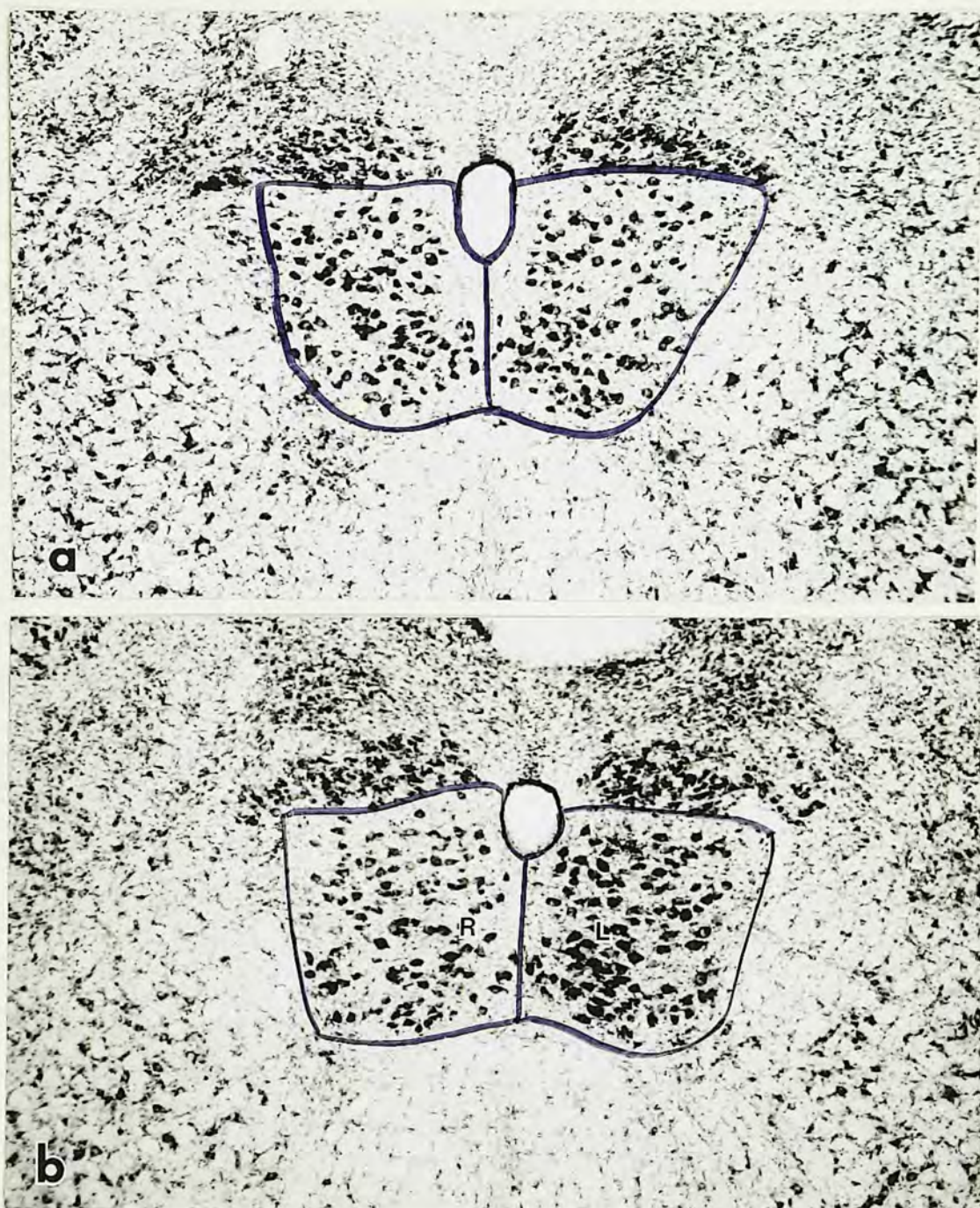


Figure 4

to 21 days following nerve injury. The basophilic staining of the perikarya of axotomized neurons, however, appeared more diffuse than observed among uninjured cells.

Light Microscopic Observations of Plastic Embedded Sections

One micron sections of hypoglossal nuclei revealed that most uninjured motoneurons contained a spherical, centrally-located nucleus, and a single prominent and densely stained nucleolus (Fig 5a). Basophilic patches of Nissl substance were observed within the perikarya of the uninjured neurons. Myelinated axons, neurites, blood vessels and some glial cells were observed in the neuropil.

Retrograde changes were apparent in the one micron sections following axotomy (Fig 5b, 6a, 6b). Seven days following axonal transection, the nuclei of some neurons were eccentrically displaced and irregularly shaped (Table 2). Cytoplasm abutting the invaginated nuclei exhibited heightened basophilia. The nucleoli of motoneurons appeared enlarged 7 days following axonal transection. Also, within the perikarya of motoneurons, the Nissl substance appeared more diffusely distributed. In the neuropil and adjacent to neurons, glial proliferation was evident. The cross-sectional profiles of neurites seemed diminished.

The retrograde response continued 13 days following axotomy. Alterations in nuclear shape and position were maximally expressed at this time, characterizing 30 to 40% of the neuronal population examined (Table 2). Nucleolar

Figure 5a. The uninjured motoneurons pictured in this one micron section of the hypoglossal nucleus from a euthyroid rat contain spherical nuclei (arrowhead) and densely stained prominent nucleoli (960X).

Figure 5b. Seven days following axotomy, the motoneurons often contained enlarged nucleoli (arrow), as shown in this photograph of a one micron section from the hypoglossal nucleus of a euthyroid animal.

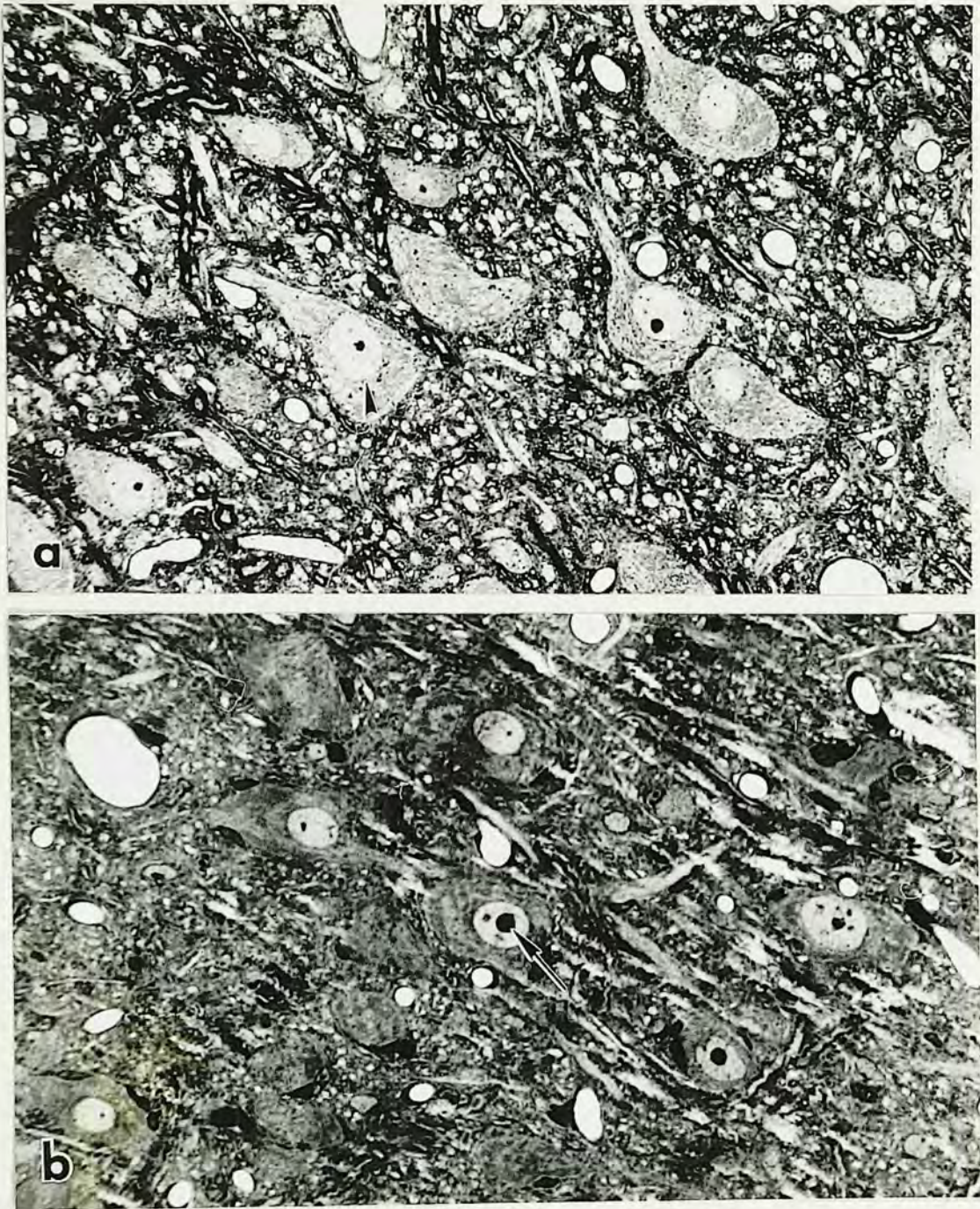


Figure 5

Figure 6a. The nucleus of a neuron, pictured in this one micron section from the hypoglossal nucleus 13 days following axotomy, demonstrates an uneven contour (arrowhead) (960X).

Figure 6b. This one micron section is taken from the hypoglossal nucleus of a euthyroid rat. Basophilic patches have reappeared in the somata of axotomized hypoglossal motoneurons 21 days following axotomy (960X).

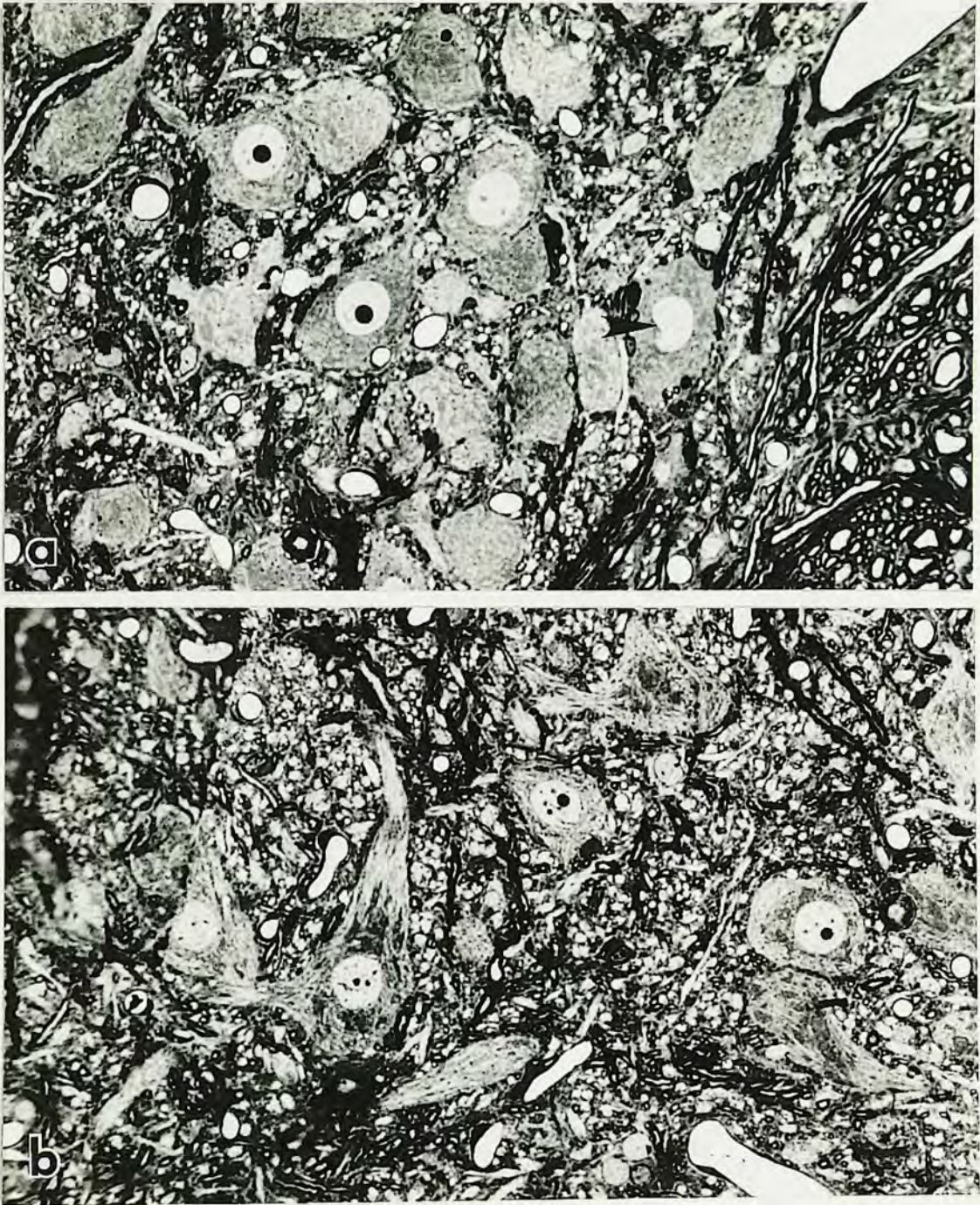


Figure 6

Table 2. Influence of Thyroid Hormone Status on the Retrograde Reaction

	7 dpa*			13 dpa			21 dpa			30 dpa		
	E	T	H	E	T	H	E	T	H	E	T	H
<u>nuclear eccentricity</u>	+	++	+++	++	++	++	0	0	0	0	0	0
<u>Infolding of the nuclear envelope and "capping"</u>	+	++	+++	++	++	++	0	0	0	0	0	0
<u>nucleolar vacuolization</u>	++	+++	++	+	++	+	0	++	0	0	++	0
<u>nuclear filaments</u>	0	0	0	0	+	+	0	+	+	0	+	0
<u>chromatolysis</u>	+++	+++	+++	++	++	++	+	+	+	0	0	0

*days post-axotomy

E - Euthyroid; T- Hyperthyroid; H - Hypothyroid

0 - no response; + + + - maximal response

enlargement, however, was no longer apparent. Twenty-one to 30 days post-axotomy, neurons regained their normal appearance.

Electron Microscopic Observations

Electron micrographs provided increased resolution of the structure of motoneurons. As observed at lower magnification, uninjured neurons contained a centrally-located nucleus with a smooth contour and a single nucleolus (Fig 7). The nucleolus was dense, compact and virtually devoid of vacuoles. Parallel stacks of elongated cisterns of the RER with interspersed polyribosomes were distributed throughout the cytoplasm, constituting the Nissl bodies observed at the light microscopic level (Fig 8). Lysosomes, mitochondria and Golgi complexes were also commonly observed in the perikarya of motoneurons.

Seven days following axotomy, nuclear eccentricity and invagination of the nuclear envelope were manifested in some of the neurons (Fig 9, 10, 11). Cytoplasm adjacent to the invaginated nuclear envelope was rich in short cisterns of RER and polyribosomes. The nucleoli within many neurons were pervaded by small vacuoles (Fig 10, 12). The long, parallel cisterns of RER were disrupted and replaced with shorter RER segments randomly arranged in the perikaryon (Fig 13). Polyribosomes seemed more abundant. Other organelles appeared unaltered.

The nuclear and perinuclear alterations first

Figure 7. The nucleus in this uninjured motoneuron, from a euthyroid animal, has a smooth contour and contains a dense, compact nucleolus (arrow). Nissl bodies, composed of elongated cisterns of RER arranged in parallel, are located within the perikaryon (4,625X).

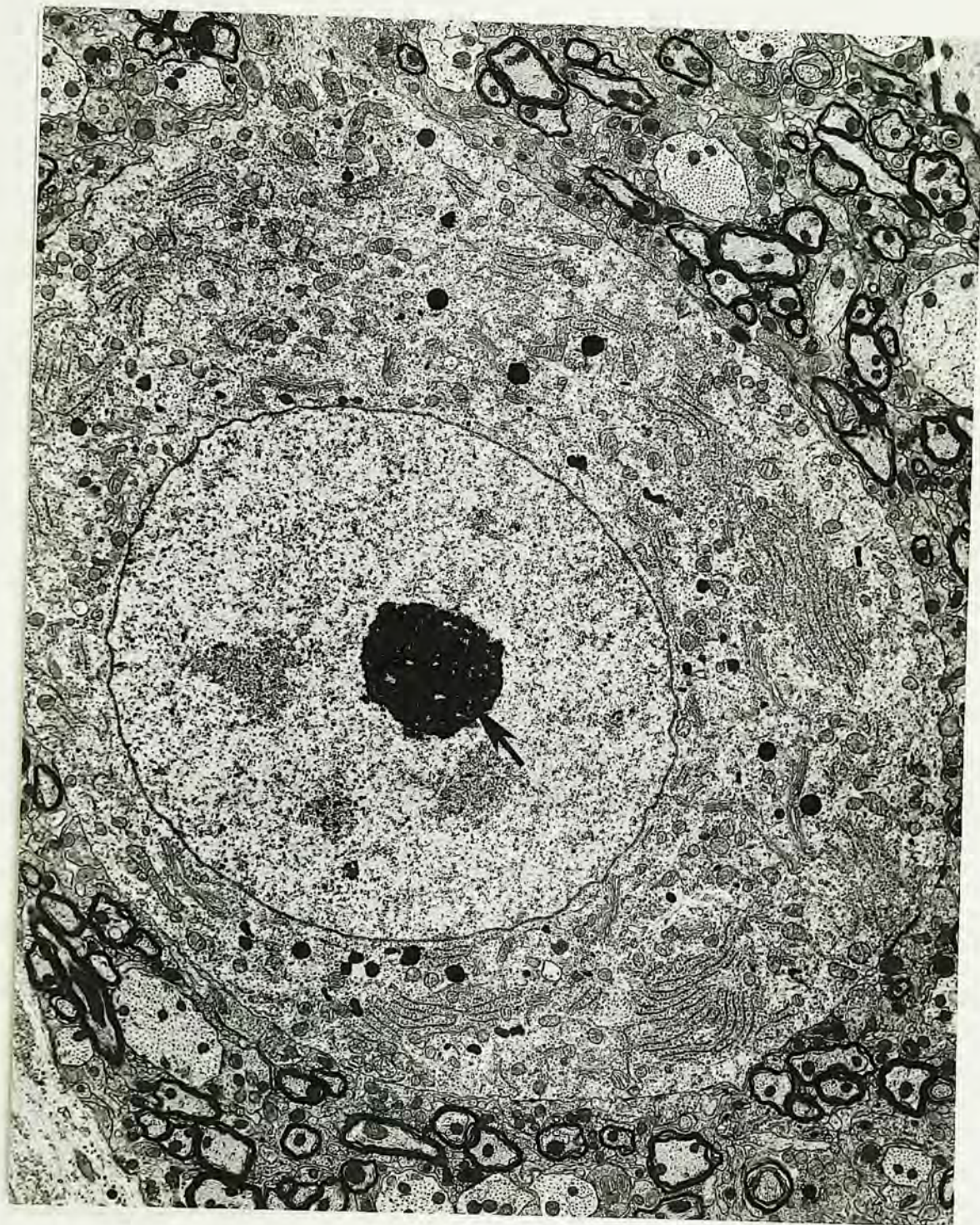


Figure 7

Figure 8. Parallel cisterns of RER (R) and interspersed polyribosomes are conspicuous in the cytoplasm of an uninjured motoneuron from a euthyroid animal. Golgi complexes (G), mitochondria (M) and lysosomes (L) are also apparent (25,740X).

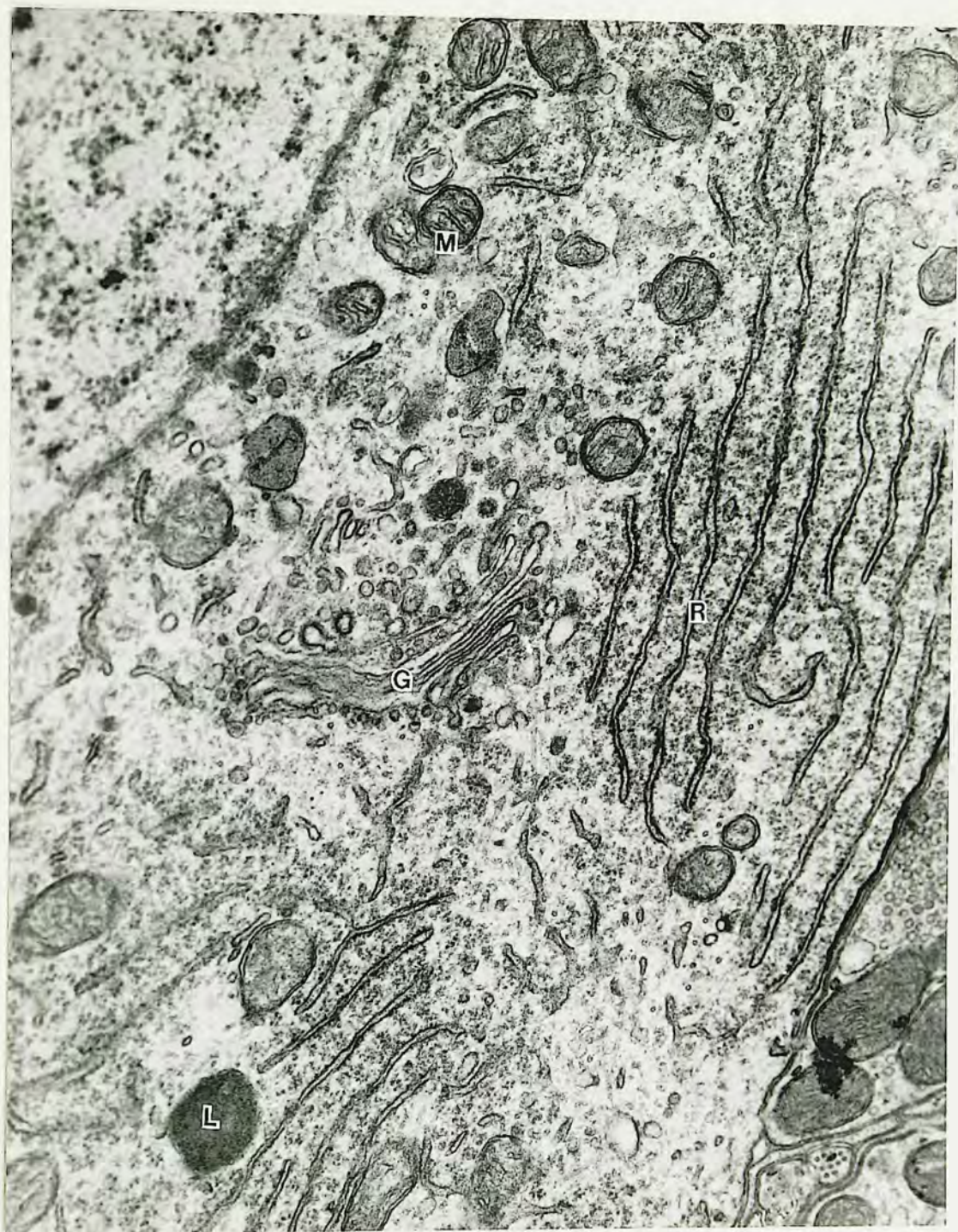


Figure 8

Figure 9. The nucleus is eccentrically displaced and the nuclear envelope invaginated in this motoneuron, from a euthyroid animal, 7 days following axotomy. The RER appears more loosely organized in the perikarya and many polyribosomes are present (8,910X).

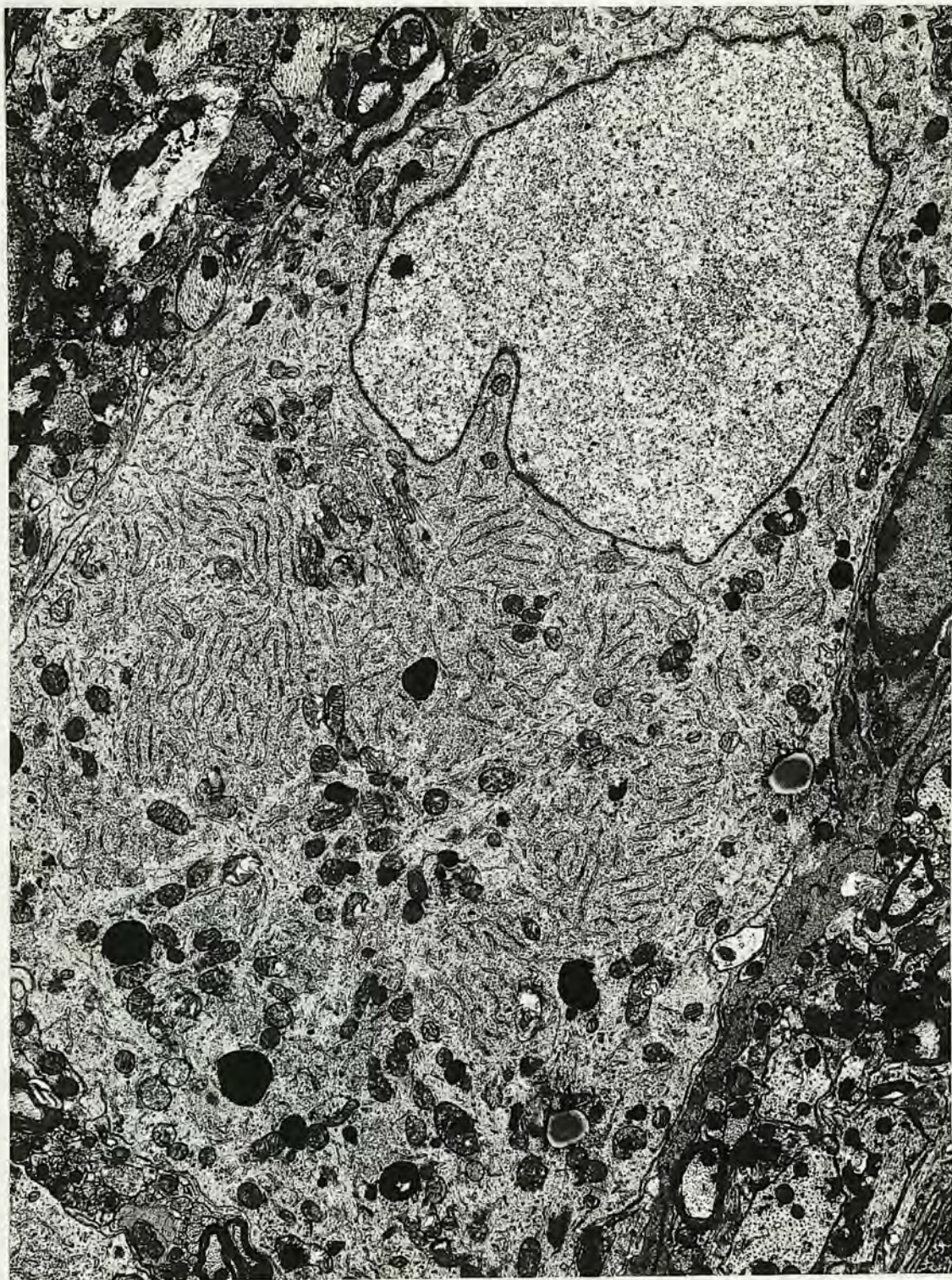


Figure 9

Figure 10. The nuclear membrane is ruffled and vacuoles pervade the nucleolus of this motoneuron, from a euthyroid animal 7 days following axotomy. Many free polyribosomes and cisterns of RER abut the invaginated nuclear envelope (arrow) (6,045X).

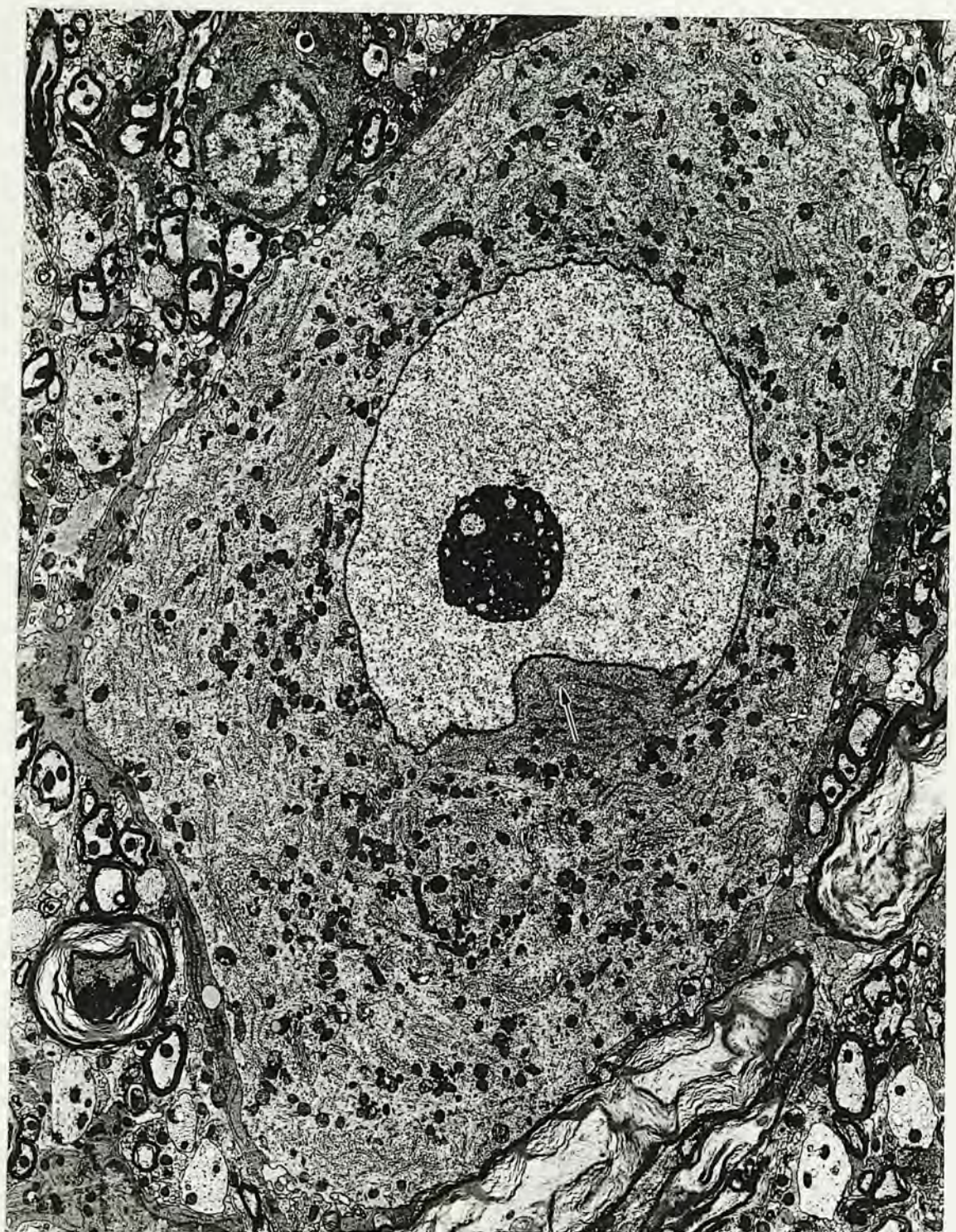


Figure 10

Figure 11. This motoneuron, from a euthyroid rat 7 days following axotomy, displays only modest invagination of the nuclear envelope (arrowhead) and RER disruption (7,920).

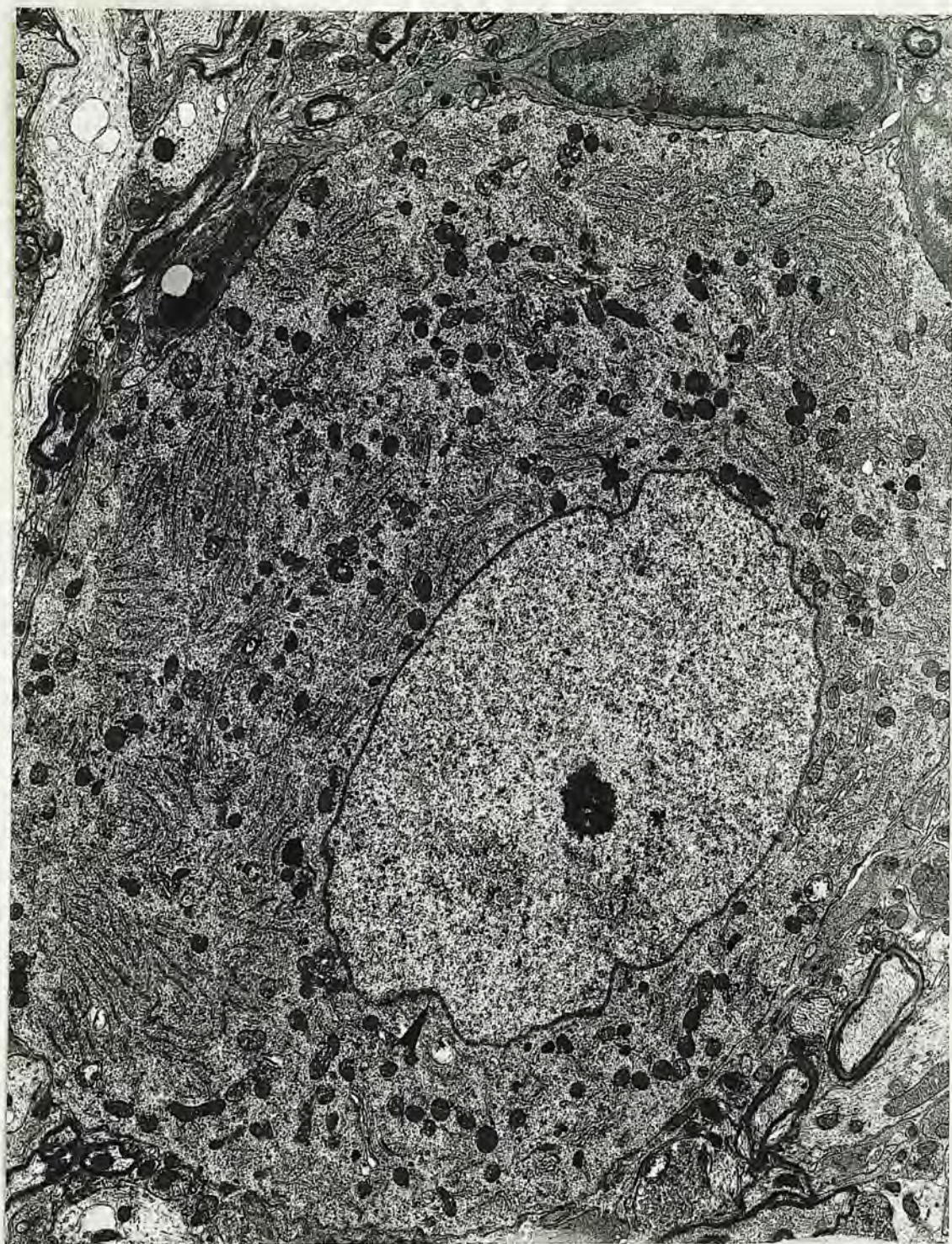


Figure 11

Figure 12. The nucleolus, of this motoneuron from a euthyroid rat 7 days post-axotomy, contains several small vacuoles. The nucleus exhibits an irregular contour (arrowhead) (6,105X).



Figure 12

Figure 13. The shortened cisterns of RER (R) are swollen and disorganized 7 days following axotomy, within this motoneuron from a euthyroid animal. The Golgi complex (G), mitochondria (M) and lysosomes (L) are not perceptively altered (25,740X).

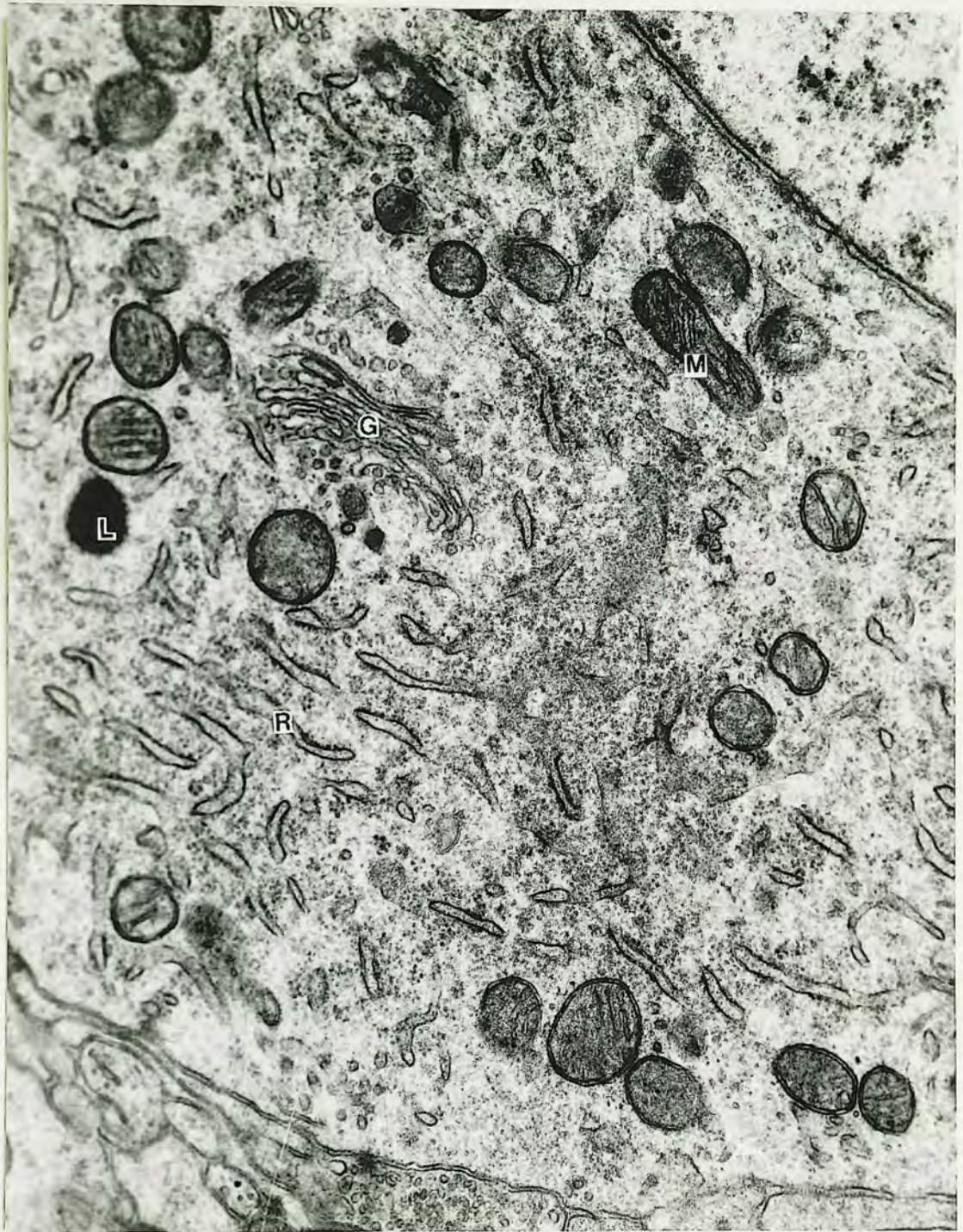


Figure 13

detected 7 days following axotomy were more frequently observed 13 days following axotomy (Fig 14, 15). Also at this time, the nucleoli of many neurons flanked the nuclear membrane. The RER, although generally disrupted, sometimes formed small parallel stacks in the perikaryon (Fig 16). Other aspects of the cell body ultrastructure remained unchanged. By 21 to 30 days following the axonal transection, the structural changes provoked by axotomy were no longer observed (Fig 17, 18).

Hypoglossal Nucleus Volume

The average volume of uninjured hypoglossal nuclei was $0.57 \pm 0.02 \text{ mm}^3$ (Fig 19a). Seven to 30 days following axotomy, the volume of the hypoglossal nucleus was significantly diminished approximately 15% ($p < 0.05$).

Neuronal Cell Body Size

All parameters which reflect neuronal cell body size indicated significant neuronal enlargement following axotomy (Table 3). Neuronal fractional volume, measured 7, 13, 21 and 30 days following axotomy, significantly exceeded ($p < 0.05$) the control estimate. The control neuronal fractional volume average of $12.8 \pm 0.6\%$ increased to a maximal value of $18.0 \pm 1.0\%$, 13 and 21 days following the nerve lesion. Total neuronal volume was significantly elevated 13 and 21 days following axonal transection ($p < 0.05$). Similarly, average neuronal cross-sectional area

Figure 14. A deep invagination of the nuclear envelope, from a motoneuron of a euthyroid animal 13 days following axotomy, is pictured. The nucleolus, with a small vacuole (arrow), is near the inner lamina of the nuclear membrane (17,500X).



Figure 14

Figure 15. This motoneuron is from a euthyroid animal 13 days following axotomy. The nucleolus (arrowhead), which contains a few small vacuoles, appears closely associated with the inner lamina of the nuclear envelope (6,000X).

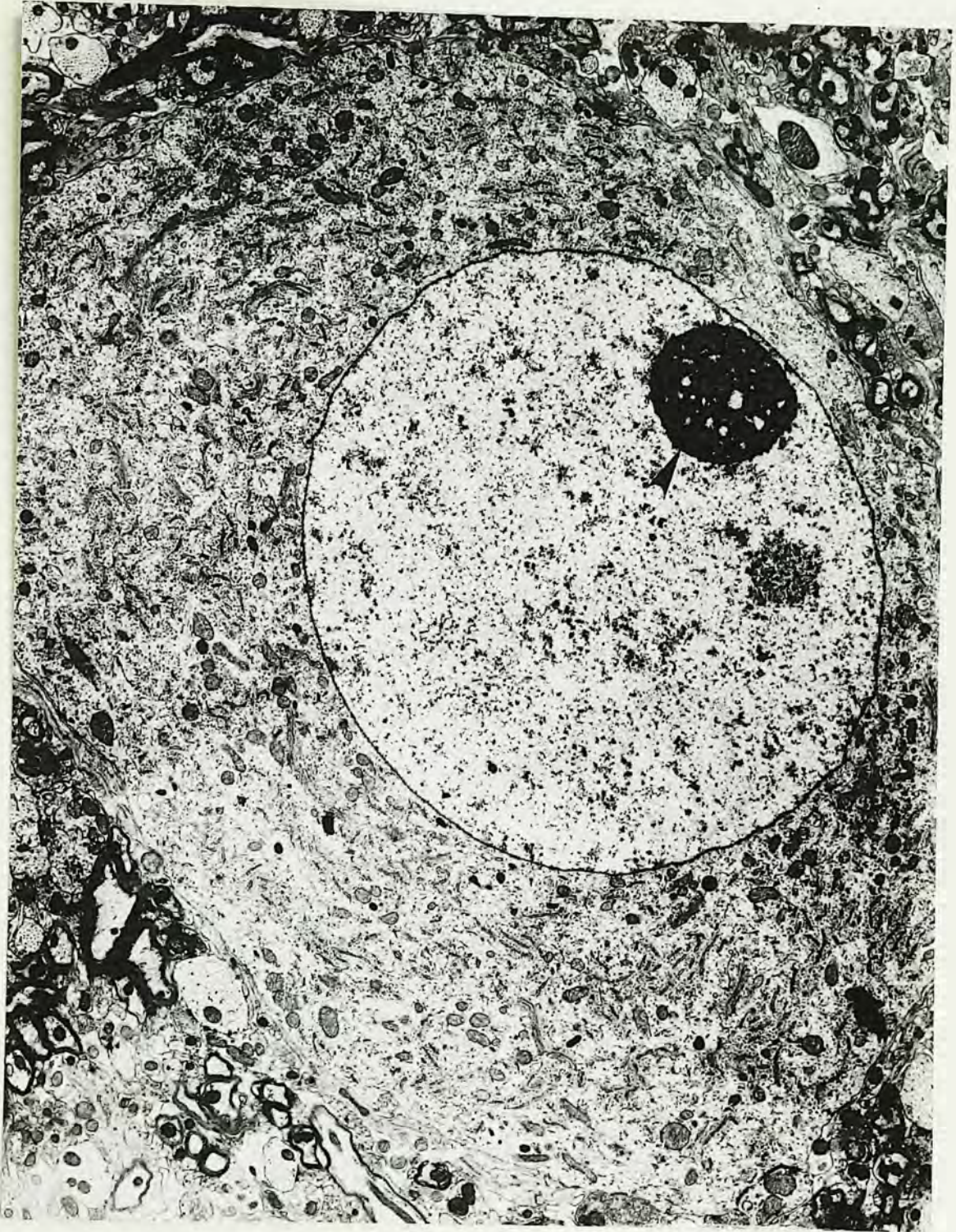


Figure 15

Figure 16. In the motoneuron perikaryon, from a euthyroid animal 13 days following axotomy, some of the cisterns of RER are organized in a parallel fashion (36,270X).

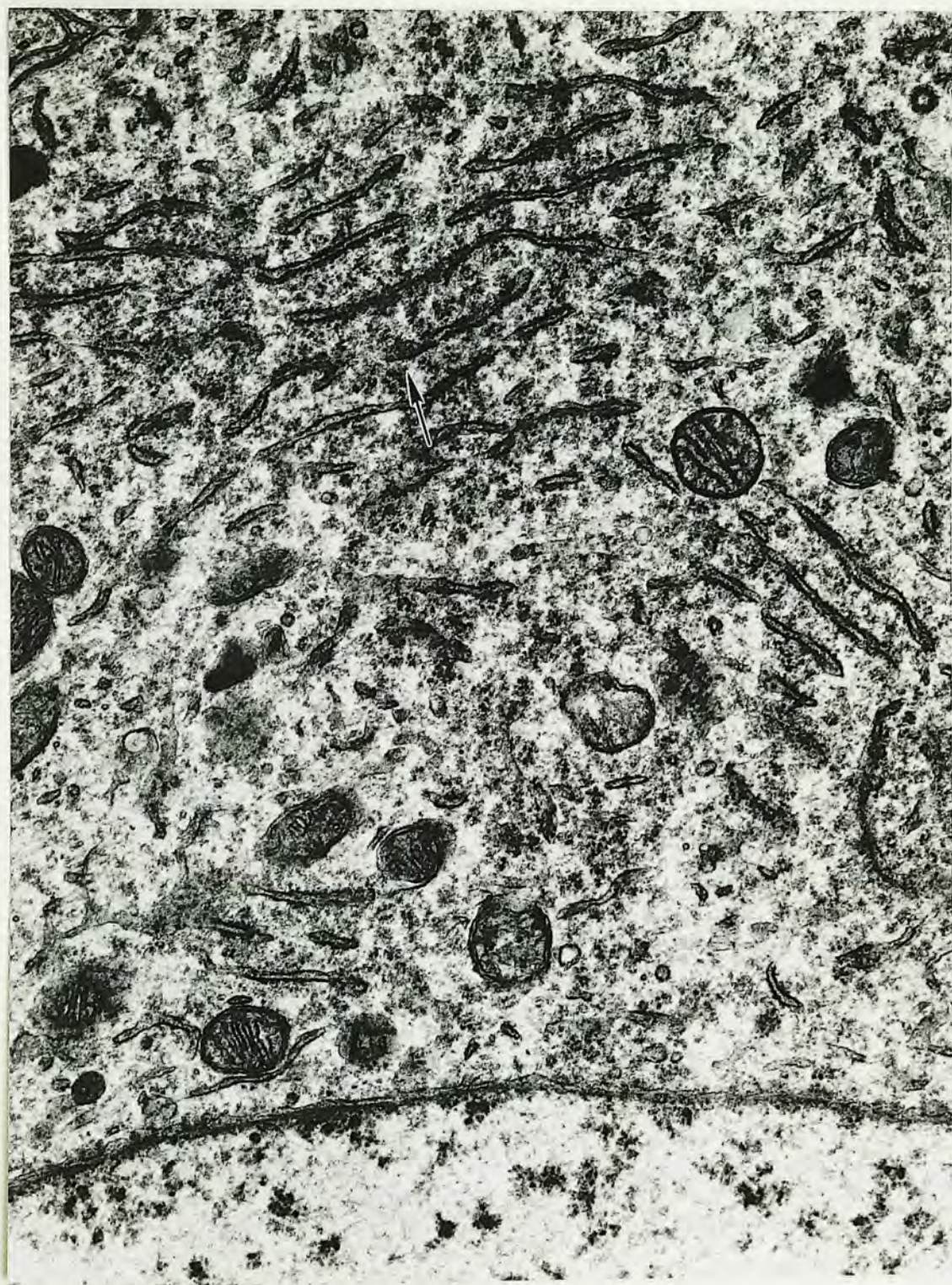


Figure 16

Figure 17. This motoneuron, from a euthyroid animal, suffered axotomy 21 days prior to fixation. The nucleus appears spherical and the nucleolus (arrow) is dense and compact, as observed among uninjured neurons (7,750X).

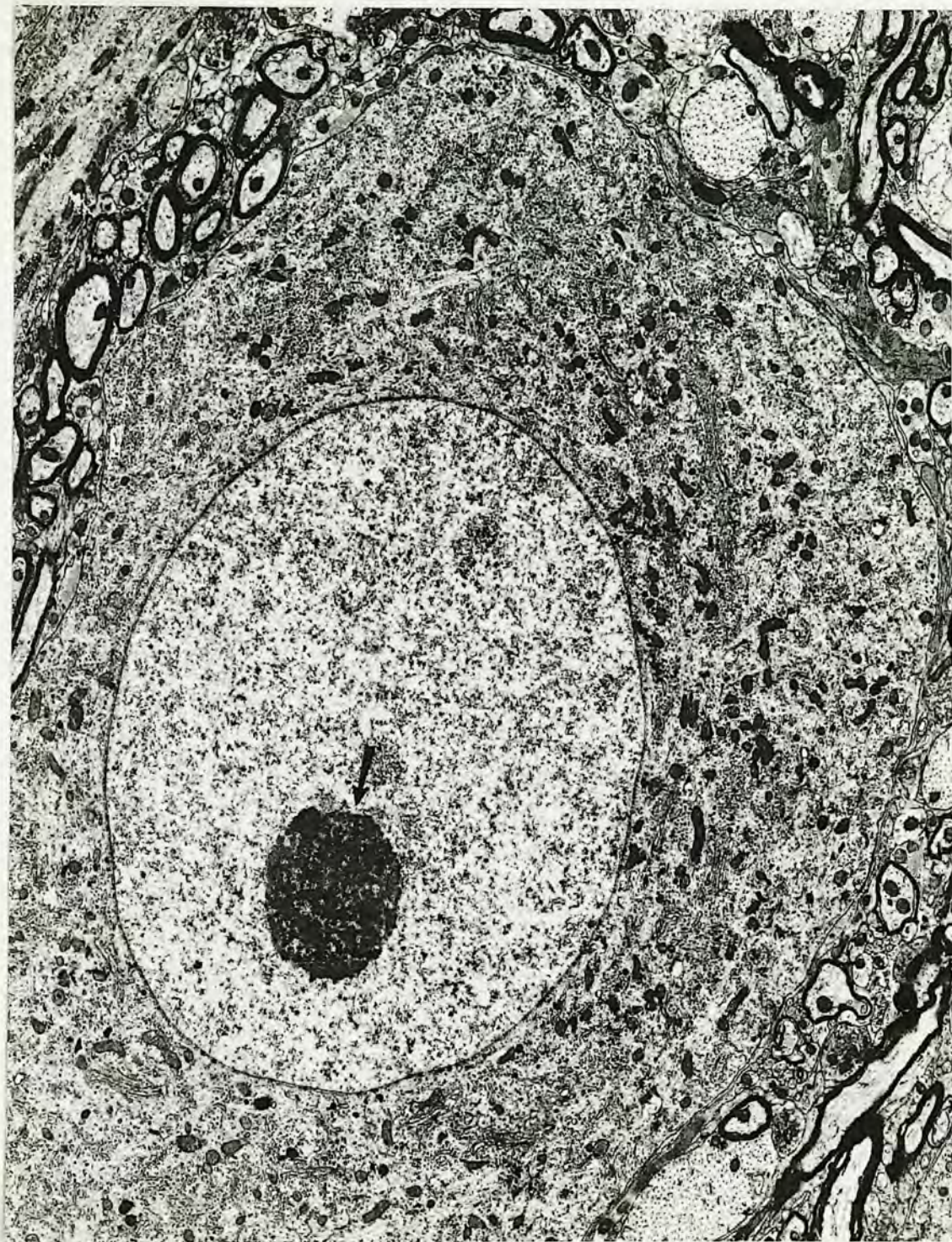


Figure 17

Figure 18. The perikaryon from a motoneuron of a euthyroid animal 30 days post-axotomy is replete with parallel cisterns of RER (R), mitochondria (M), Golgi complexes (G) and lysosomes (L) (30,420X).

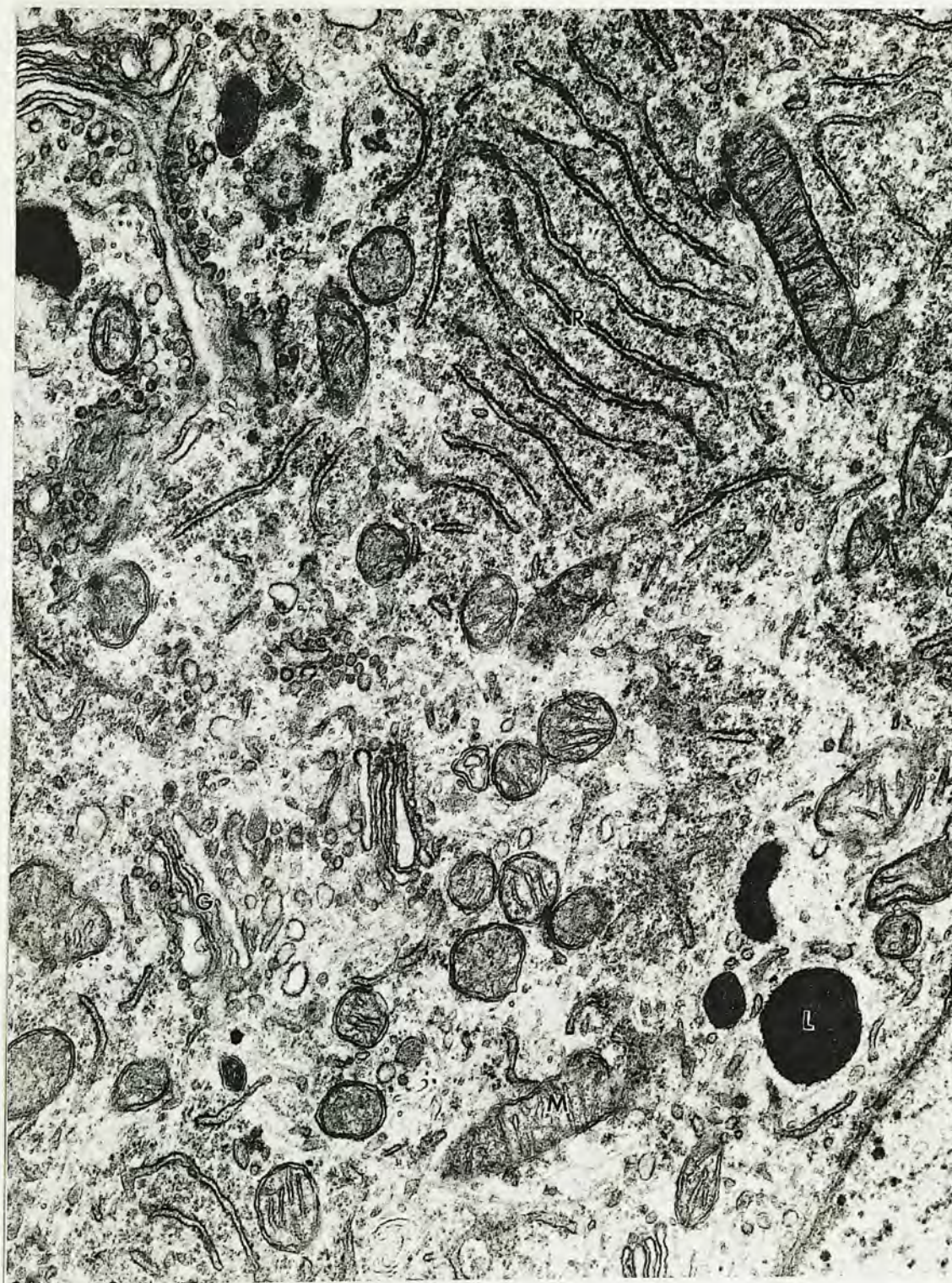


Figure 18

Figure 19. Mean (\pm SEM) values for hypoglossal (XIIth) nucleus volume at different intervals following axotomy among different thyroid hormone treatment groups (A - euthyroid, B - hyperthyroid, C - hypothyroid). Within each treatment group, bars with different superscripts are significantly different ($p < 0.05$).

Hypoglossal Nucleus Volume Following Axotomy

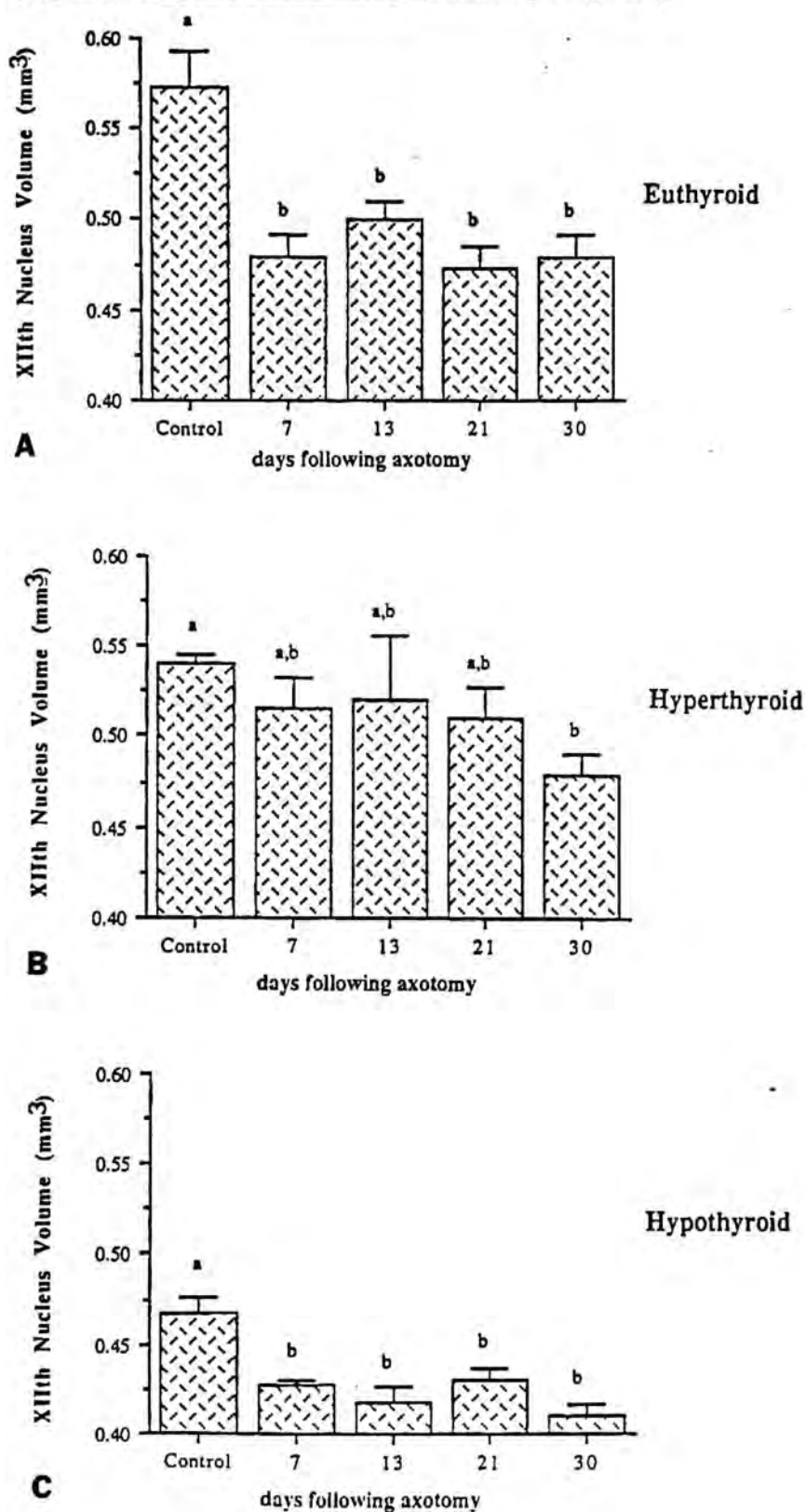


Figure 19

TABLE 3. Neuronal Size Following Axotomy^{a,b}

EUTHYROID

	Neuronal Fractional Volume (%)	Total Neuronal Volume (mm ³)	Neuronal XS-area (μm ²)	Neuronal Maximal Diameter (μm)
Control	12.8 ± 0.6 ^d	0.074 ± 0.006 ^d	528.1 ± 7.5 ^d	31.8 ± 0.3 ^d
7dpa ^c	16.7 ± 1.2 ^e	0.080 ± 0.007 ^{d,e}	568.6 ± 13.5 ^d	34.1 ± 0.9 ^{d,e}
13dpa	18.0 ± 1.0 ^e	0.090 ± 0.006 ^e	603.7 ± 6.2 ^{d,e}	36.7 ± 3.2 ^e
21dpa	18.0 ± 0.6 ^e	0.085 ± 0.002 ^e	652.8 ± 12.2 ^e	35.7 ± 0.3 ^e
30dpa	16.7 ± 0.3 ^e	0.081 ± 0.002 ^{d,e}	580.9 ± 6.5 ^{d,e}	32.3 ± 0.4 ^d

HYPERTHYROID

	Neuronal Fractional Volume (%)	Total Neuronal Volume (mm ³)	Neuronal XS-area (μm ²)	Neuronal Maximal Diameter (μm)
Control	13.7 ± 0.6 ^d	0.073 ± 0.005 ^d	534.7 ± 4.6 ^d	30.3 ± 0.6 ^d
7dpa	16.0 ± 0.5 ^e	0.082 ± 0.003 ^{d,e}	557.3 ± 13.6 ^d	32.9 ± 0.3 ^{d,e}
13dpa	17.0 ± 1.0 ^e	0.088 ± 0.002 ^e	616.4 ± 28.2 ^{d,e}	34.7 ± 0.9 ^e
21dpa	18.0 ± 0.5 ^e	0.092 ± 0.002 ^{e,f}	650.0 ± 11.3 ^{e,f}	34.7 ± 0.8 ^e
30dpa	20.3 ± 0.3 ^f	0.097 ± 0.003 ^f	692.9 ± 23.2 ^f	35.7 ± 0.8 ^e

HYPOTHYROID

	Neuronal Fractional Volume (%)	Total Neuronal Volume (mm ³)	Neuronal XS-area (μm ²)	Neuronal Maximal Diameter (μm)
Control	14.3 ± 0.3 ^d	0.067 ± 0.002 ^d	537.3 ± 16.5 ^d	31.5 ± 1.0 ^d
7dpa	17.7 ± 0.7 ^e	0.075 ± 0.005 ^d	556.4 ± 34.0 ^d	34.0 ± 0.3 ^d
13dpa	16.3 ± 1.3 ^e	0.068 ± 0.007 ^d	506.4 ± 28.1 ^d	32.1 ± 1.1 ^d
21dpa	16.7 ± 0.3 ^e	0.072 ± 0.001 ^d	517.4 ± 7.5 ^d	33.2 ± 0.1 ^d
30dpa	17.0 ± 1.2 ^e	0.070 ± 0.004 ^d	528.3 ± 25.0 ^d	32.3 ± 1.4 ^d

^aValues are means ± SEM^bValues within columns with different superscripts are significantly different (p<0.05)^cdays post-axotomy

and maximal diameter demonstrated a significant increase, from the control average, 21 days post-axotomy and 13 to 21 days post-axotomy, respectively. By 30 days following the axonal lesion, total neuronal volume, neuronal cross-sectional area and maximal diameter evidenced average values not significantly different from the uninjured controls.

Volume and Surface Area of the Cell Nucleus

Parameters estimating the volume and surface area of nuclei within the motoneurons demonstrated no significant change following axotomy. Nuclear fractional volume averaged $25.2 \pm 1.4\%$ among all euthyroid animals (Table 4) and nuclear surface density averaged $1,079.4 \pm 71.8/\text{cm}$ (Fig 20a). The total nuclear volume was $0.019 \pm 0.003 \text{ mm}^3$. Total

Table 4. Cell Nucleus Volume

Thyroid Hormone Status	Nuclear Fractional Volume (%)	Total Nuclear Volume (mm^3)
Euthyroid	25.2 ± 1.4	0.019 ± 0.003
Hyperthyroid	22.2 ± 0.9	0.018 ± 0.002
Hypothyroid	23.6 ± 0.7	0.017 ± 0.001

nuclear surface area, which averaged $8.3 \pm 0.8 \text{ mm}^2$, demonstrated a slight, though not significant, post-axotomy increase (Fig 20a).

Nucleolar Volume

Nucleolar fractional volume increased nearly 300% 7

Figure 20. Nuclear surface density and surface area from eu- (A), hyper- (B) and hypothyroid (C) animals following axotomy. Means (\pm SEM) are reported. Within treatment groups, points with different superscripts are significantly different ($p < 0.05$).

Nuclear Surface Density and Area Following Axotomy

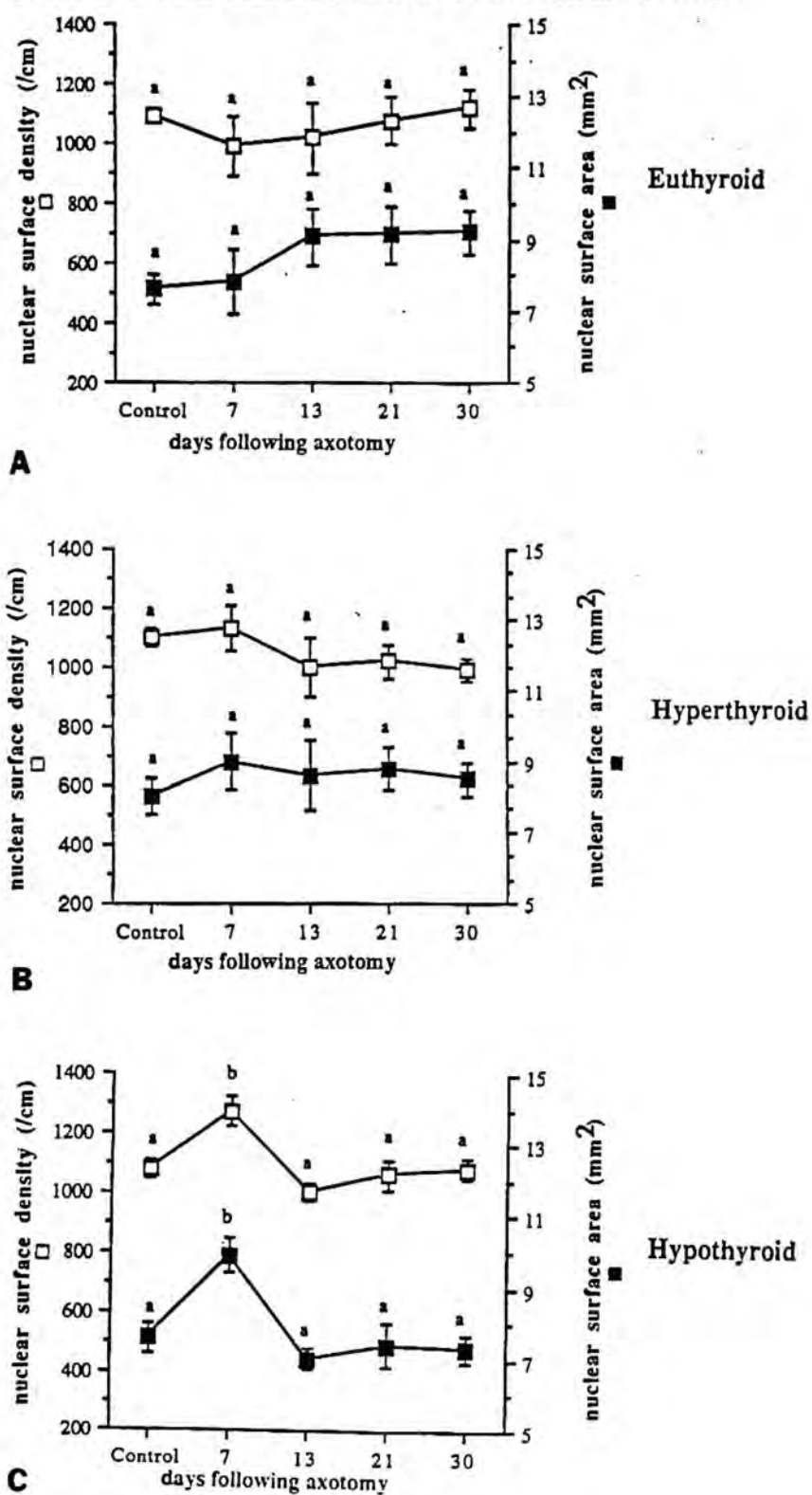


Figure 20

days following axotomy from $2.4 \pm 0.6\%$ to $6.9 \pm 1.3\%$ (Fig 21a). Thirteen to 30 days following axotomy, the nucleolar fractional volume was not significantly different from the control average, however it appeared to be slightly elevated. Total nucleolar volume increased significantly ($p < 0.05$) 7 days following axonal transection, from an average of $3.96 \times 10^5 \text{ um}^3$ to $1.35 \times 10^6 \text{ um}^3$ (Fig 22a). Total nucleolar volume demonstrated a persistent, though not significant ($p > 0.05$), elevation 13 to 30 days following axotomy.

Surface Area and Volume of Perikaryal Organelles

The RER surface area was significantly diminished following axonal transection. Among the uninjured population, RER surface density averaged $40,453 \pm 917/\text{cm}$ and total RER surface area was $220 \pm 8 \text{ mm}^2$ (Fig 23, 24). One week following axotomy the RER surface density and total surface area were reduced by 20-30% ($p < 0.05$). Control RER surface density and surface area levels were reestablished 21 and 13 days following axotomy, respectively.

Smooth membrane surface density, averaging $2,095.3 \pm 200.5/\text{cm}$ among the controls, increased more than two-fold one week following axotomy to $5,217 \pm 188/\text{cm}$ (Fig 25). Thirty days post-axotomy, the smooth membrane surface density was not significantly different from that of the uninjured controls. Total surface area of the smooth membrane paralleled the response pattern of the surface

Figure 21. Nucleolar fraction volume from eu- (A), hyper- (B) and hypothyroid (C) animals following axotomy. Means (\pm SEM) are reported. Within treatment groups, points with different superscripts are significantly different ($p < 0.05$).

Nucleolar Fractional Volume Following Axotomy

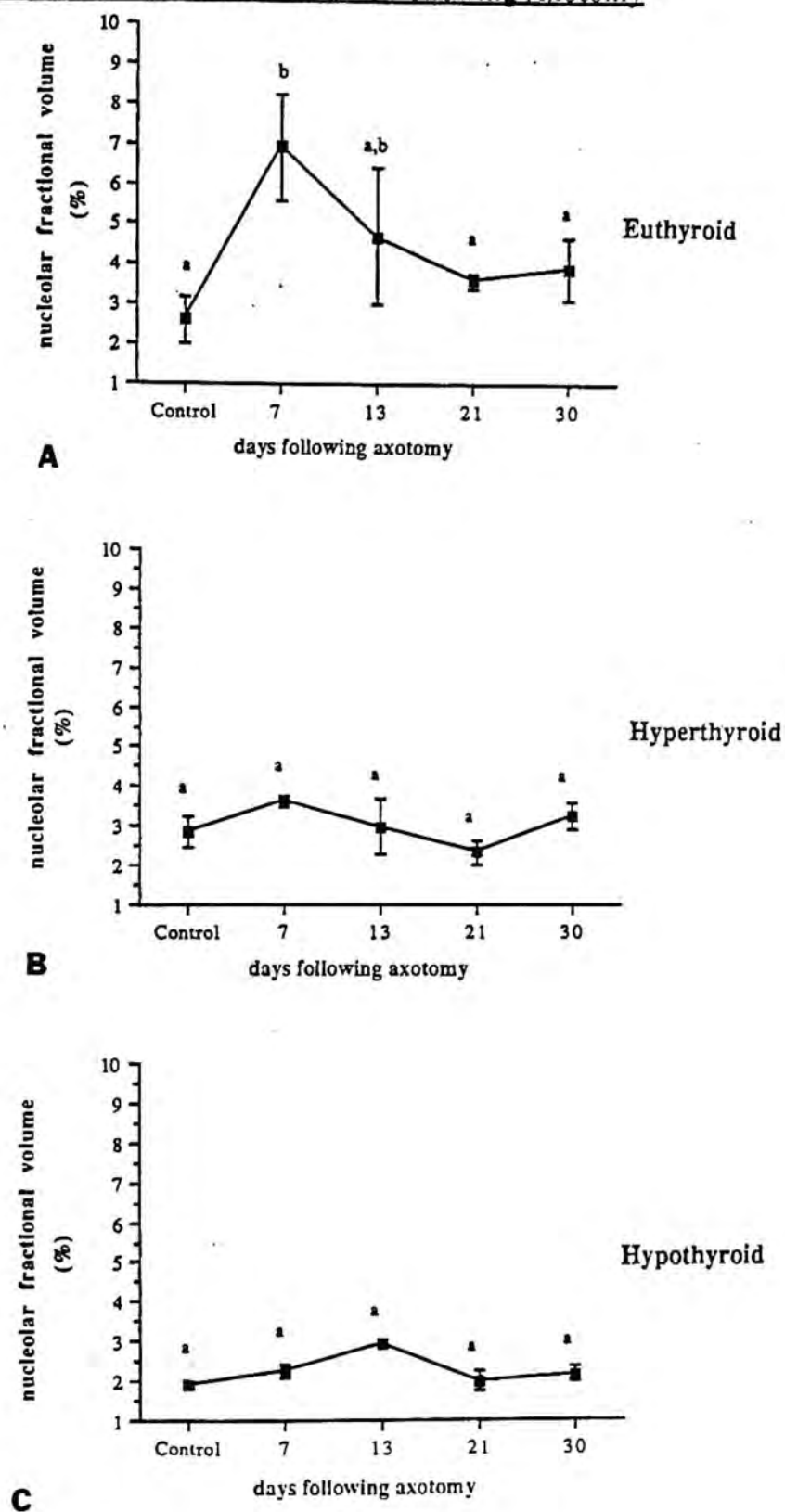


Figure 21

Figure 22. Mean (\pm SEM) values for total nucleolar volume among eu- (A), hyper- (B) and hypothyroid (C) animals following axotomy. Within treatment groups, bars with different superscripts are significantly different ($p < 0.05$).

Total Nucleolar Volume Following Axotomy

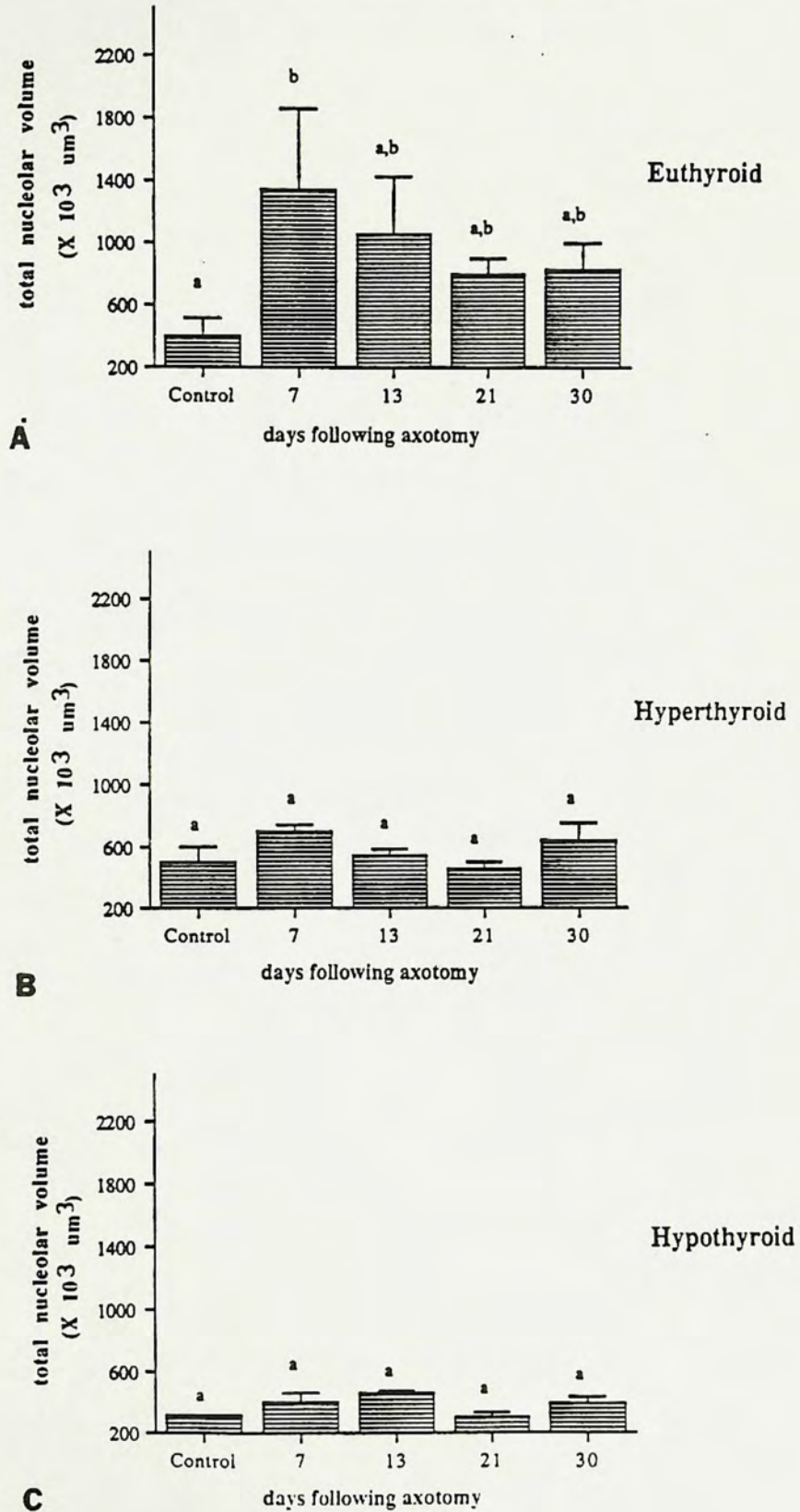


Figure 22

Figure 23. Mean (\pm SEM) RER surface density per neuronal cell body from euthyroid animals at different intervals following hypoglossal nerve transection. Points with different superscripts are significantly different ($p < 0.05$).

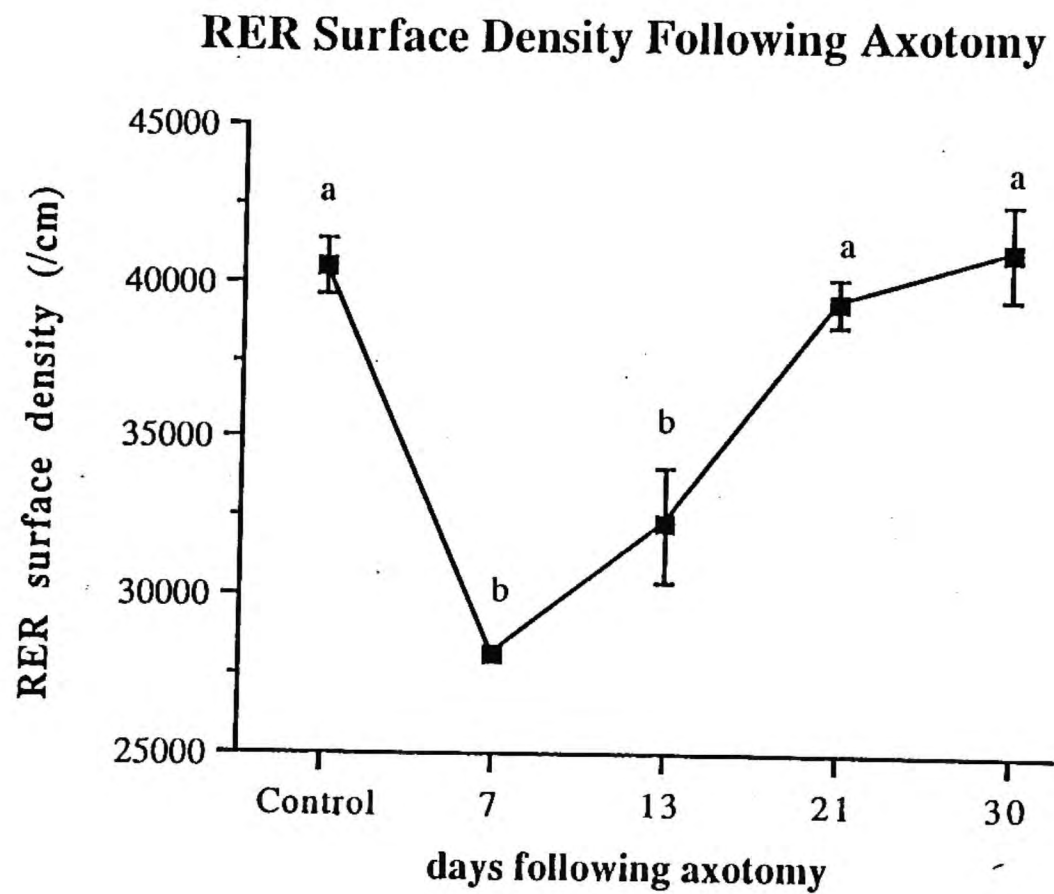


Figure 23

Figure 24. Total RER surface area (mean \pm SEM) within motoneurons of the hypoglossal nucleus from euthyroid animals following axotomy. Different superscripts above bars denote significant differences ($p < 0.05$).

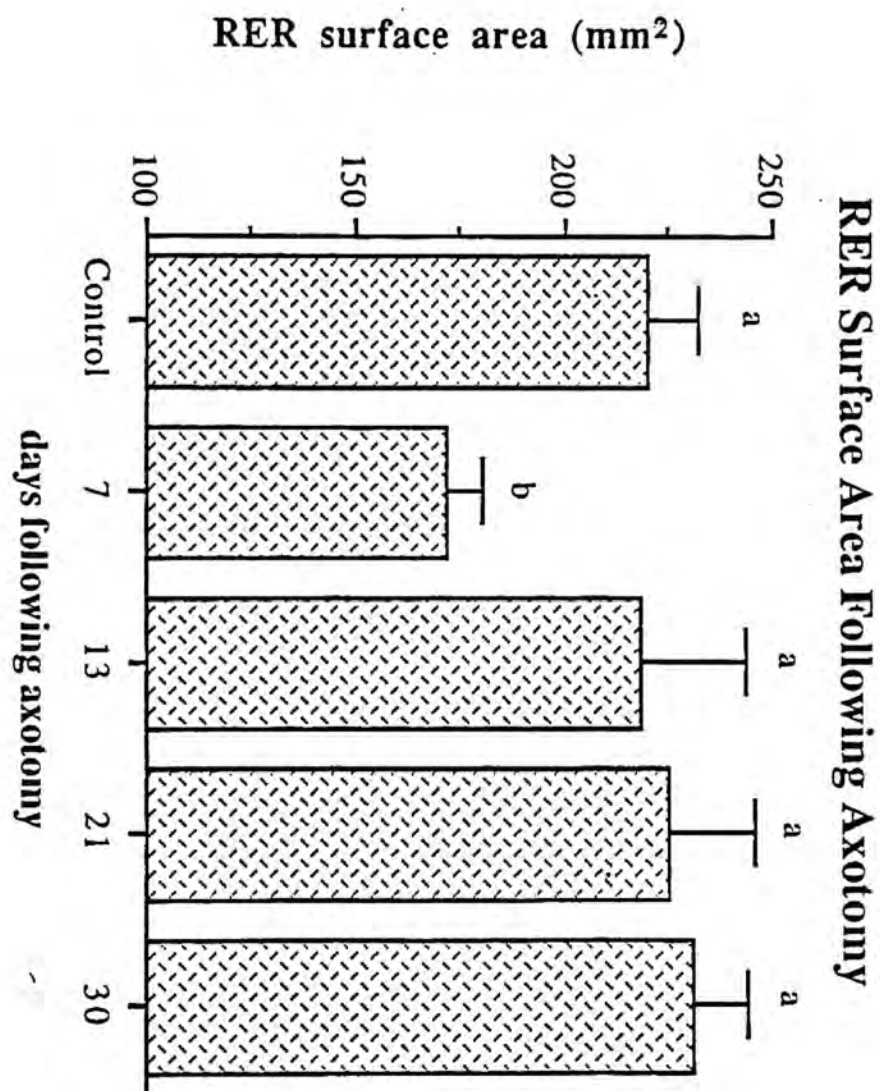


Figure 24

Figure 25. Mean (\pm SEM) non-Golgi smooth membrane surface density within the perikaryon of uninjured and axotomized hypoglossal motoneurons from euthyroid animals. Points with different superscripts are significantly different ($p < 0.05$).

Smooth Membrane Surface Density Following Axotomy

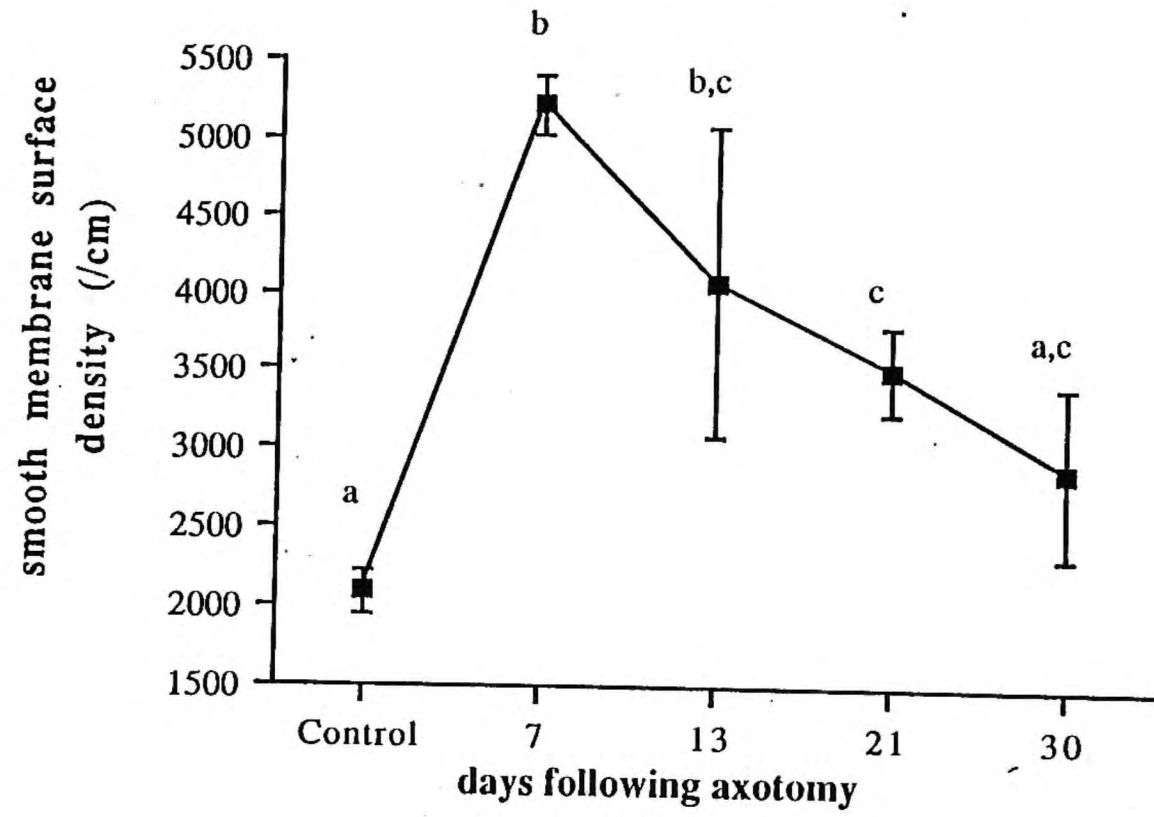


Figure 25

density (Fig 26); the smooth membrane component of the cytoplasm more than doubled its surface area 7 days following axotomy. Three to 4 weeks following axonal transection, smooth membrane surface area was not significantly different from the control measurements.

Other organelles within the perikarya of the motoneurons were not significantly altered following axotomy. The Golgi apparatus surface density and total surface area averaged $22,891 \pm 673/\text{cm}$ and $137 \pm 10 \text{ mm}^2$, respectively (Fig 27). Both, Golgi apparatus surface density and area, slightly increased following axotomy. Mitochondrial fractional volume and total volume averaged $8.0 \pm 0.2\%$ and $4.7 \pm 0.3 \times 10^6 \text{ um}^3$, respectively (Fig 28). Finally, lysosomal fractional volume averaged $0.88 \pm 0.04\%$ and total volume averaged $5.0 \pm 0.2 \times 10^7 \text{ um}^3$ (Fig 29).

THYROID HORMONE STATUS OF ANIMALS

Total plasma thyroxine averaged $4.1 \pm 0.2 \text{ ug/dl}$ among the euthyroid animals. Among the hyperthyroid animals, total serum thyroxine averaged $44.3 \pm 16.4 \text{ ug/dl}$; the lowest thyroxine value detected among the hyperthyroid animals was 17.0 ug/dl . Each hypothyroid animal yielded a total serum thyroxine level less than the sensitivity of the assay, 1.0 ug/dl . Body weight increased with age among the eu- and hyperthyroid animals (Fig 30). In contrast, the average body weight of hypothyroid animals was constant at all times analyzed.

Figure 26. Non-Golgi smooth membrane surface area (mean \pm SEM) within motoneurons of the hypoglossal nuclei from euthyroid animals following axotomy. Different superscripts above bars denote significant differences ($p < 0.05$).

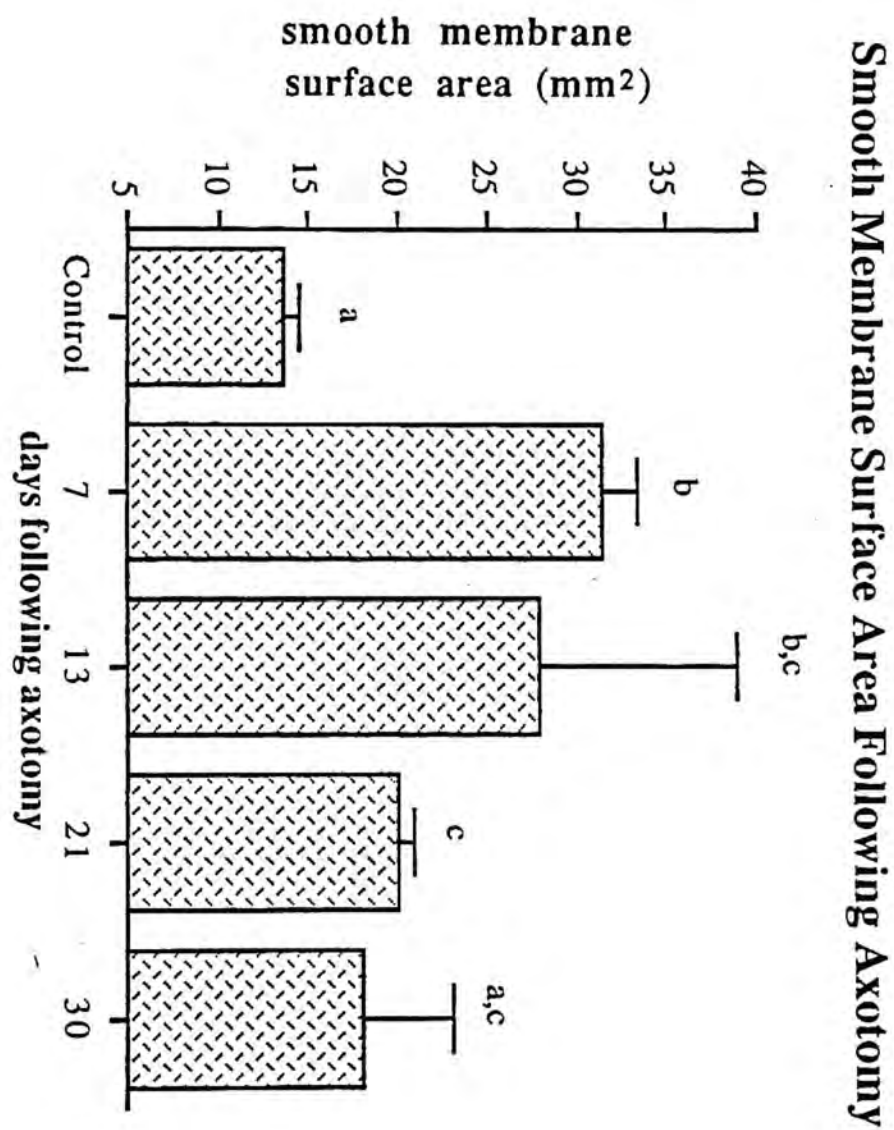


Figure 26

Figure 27. Golgi apparatus surface density and total surface area within uninjured and axotomized hypoglossal motoneurons from euthyroid animals. Means (\pm SEM) are reported. Within parameters, points with different superscripts are significantly different ($p < 0.05$).

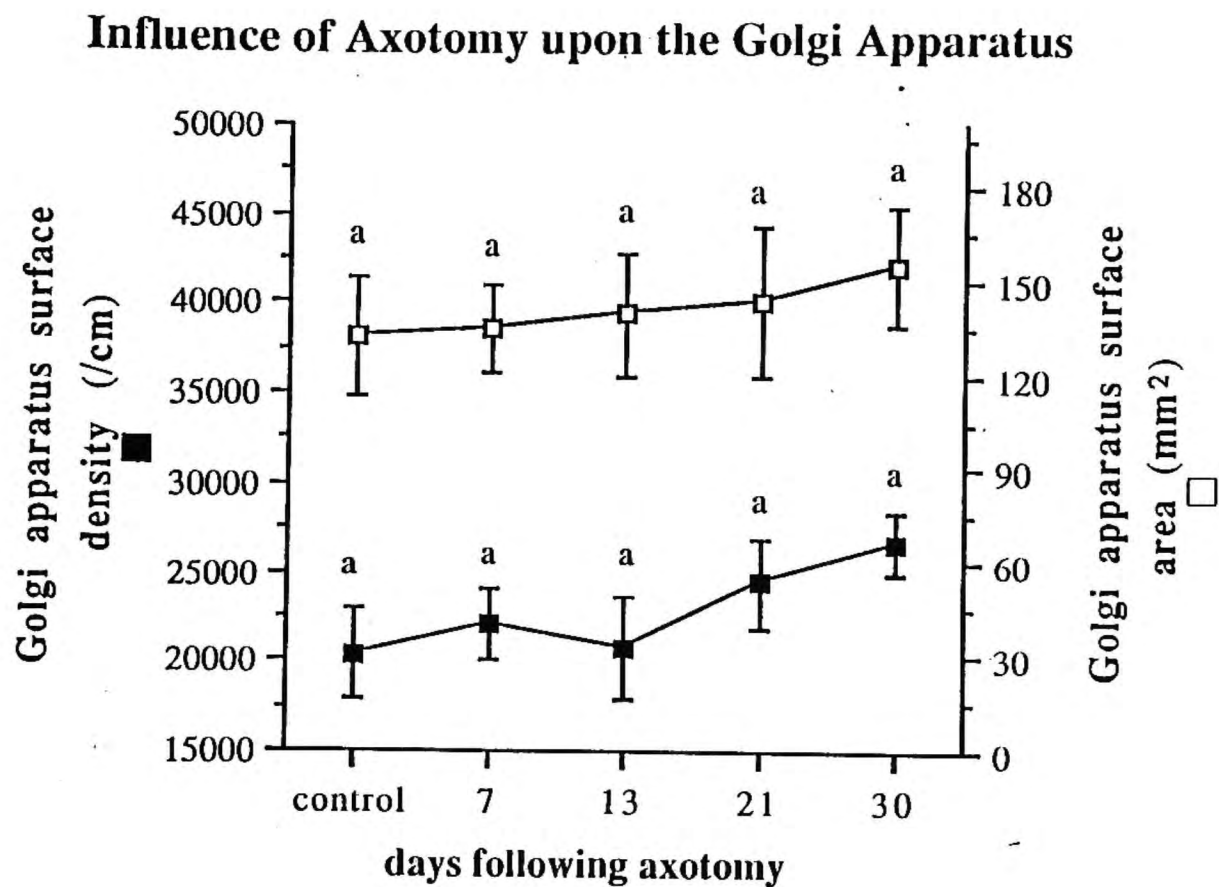


Figure 27

Figure 28. Mean values (\pm SEM) for mitochondrial fractional volume and total volume among uninjured and axotomized hypoglossal motoneurons from euthyroid animals. Within parameters, points with different superscripts are significantly different ($p < 0.05$).

Influence of Axotomy upon Mitochondria

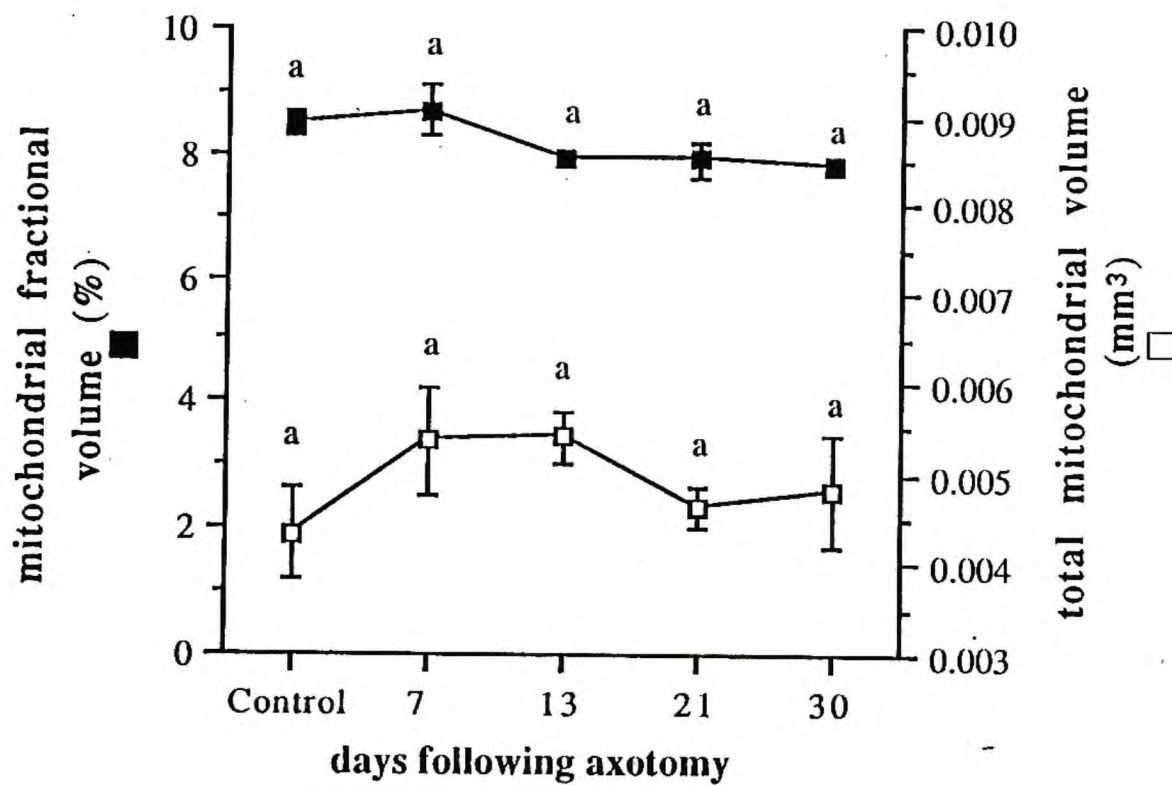


Figure 28

Figure 29. Lysosomal fractional volume and total volume among hypoglossal motoneurons from euthyroid animals following axotomy. Within parameters, different superscripts denote significant differences among means (\pm SEM).

Influence of Axotomy upon Lysosomes

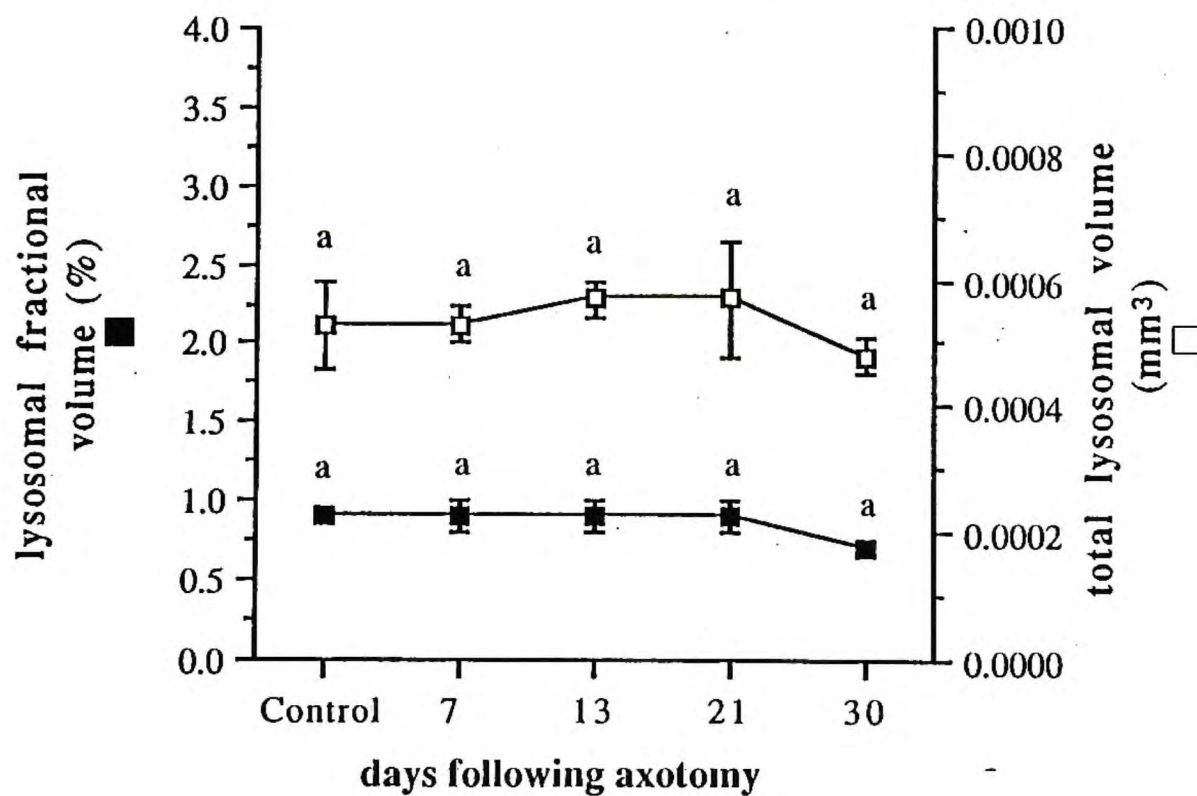


Figure 29

Figure 30. Mean body weights of eu-, hyper- and hypothyroid animals included in this study.

Body Weight of Eu-, Hyper- and Hypothyroid Animals

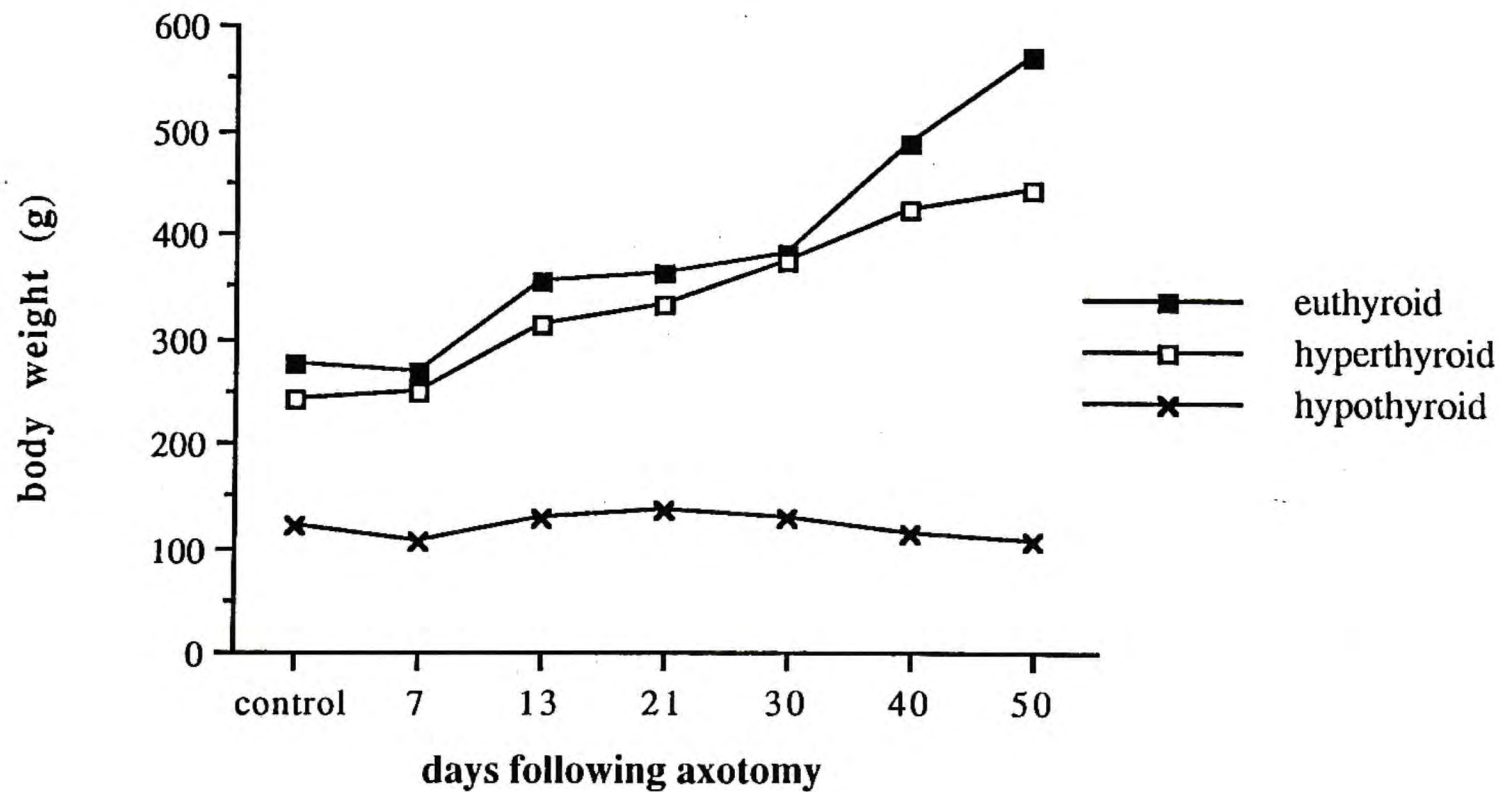


Figure 30

THE RETROGRADE RESPONSE AMONG THE HYPERTHYROID POPULATION

Light Microscopic Observations of Vibratome Sections

The uninjured hypoglossal nuclei, as viewed in 50 microns sections, were not detectably influenced by the hyperthyroid state induced in this study. Multipolar motoneurons as well as some glial cells, blood vessels and neurites were present in the hypoglossal nucleus (Fig 31a). Among the hyperthyroid animals, the retrograde response to axotomy was generally similar to that observed in the euthyroid group. Following axotomy, the cross-sectional profile of hypoglossal nuclei appeared unchanged or diminished (Fig 31b). Neuronal enlargement and heightened basophilic staining of neurons were evident 7 to 30 days following nerve transection.

Light Microscopic Observations of Plastic Embedded Sections

In one micron sections, the uninjured motoneurons and surrounding neuropil from hyperthyroid animals were indistinguishable from their counterparts among the euthyroid population (Fig 32a). The retrograde response to axotomy exhibited by motoneurons from hyperthyroid animals generally resembled that of the euthyroid population, with a few exceptions. Nuclear eccentricity and "ruffling" of the nuclear envelope were observed in approximately 40% of the neurons examined 7 days following axotomy (Fig 32b). The nuclear changes were less prevalent 13 days following

Figure 31a. The uninjured hypoglossal nuclei (outlined) from a hyperthyroid rat are similar in appearance to those of the euthyroid population. Basophilic neurons within the hypoglossal nucleus can be seen in this photograph of a 50 micron section from the medulla (48X).

Figure 31b. A 50 micron section from the medulla of a hyperthyroid rat 13 days following left hypoglossal neurotomy. Although the cross-sectional profiles of the injured (L) versus uninjured (R) hypoglossal nuclei appear similar, the injured neurons demonstrate enlargement (48X).

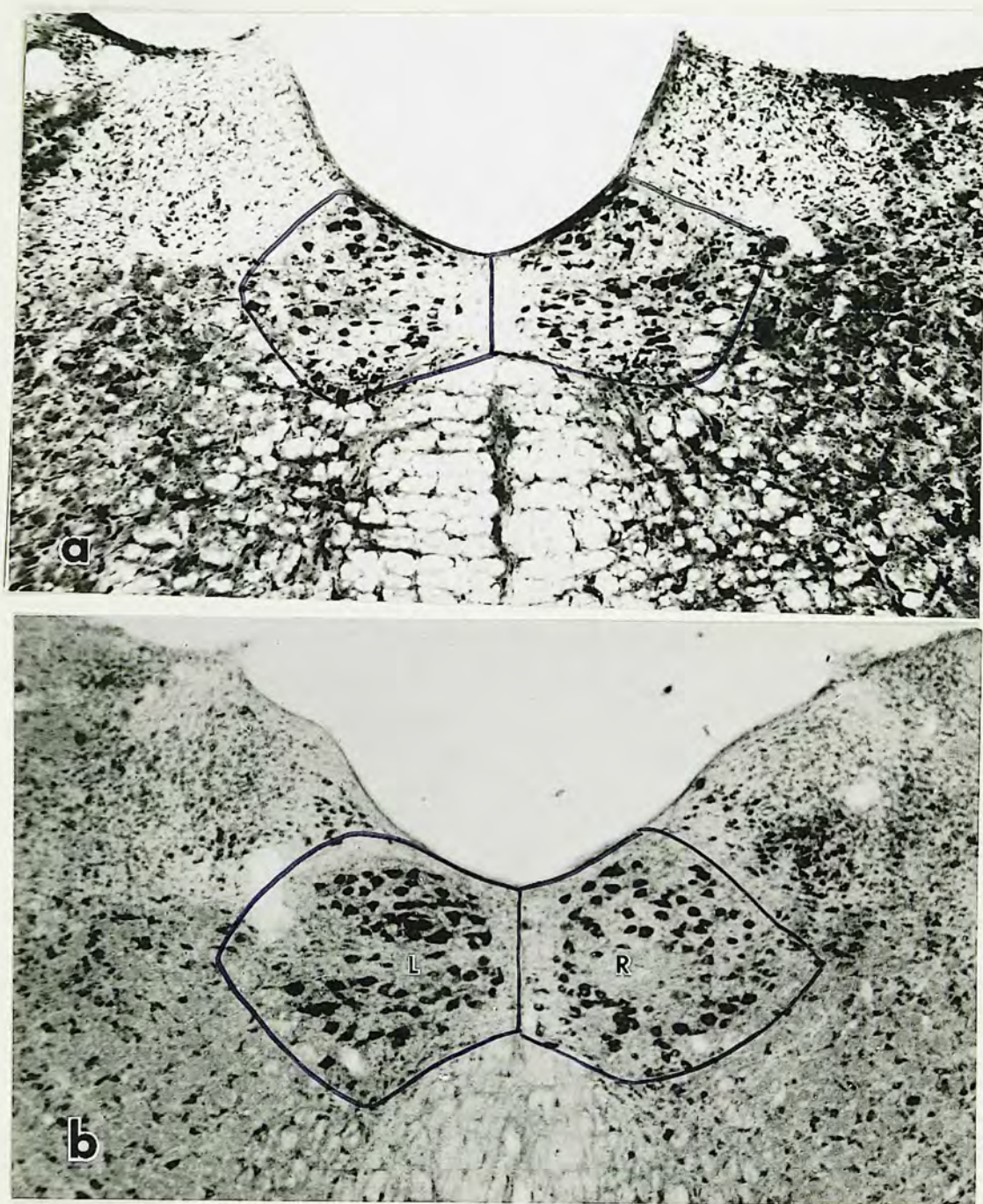


Figure 31

Figure 32a. This is a one micron section from an uninjured hypoglossal nucleus of a hyperthyroid rat. The motoneurons contain spherical nuclei, and the nucleolus is densely stained (1,000X).

Figure 32b. Thirty days following axotomy, a typical motoneuron from the hyperthyroid population, as pictured in this one micron section of the hypoglossal nucleus, contains a nucleolus with an enlarged vacuole (arrow). Reformed Nissl bodies are present in the perikarya of many neurons (1,000X).

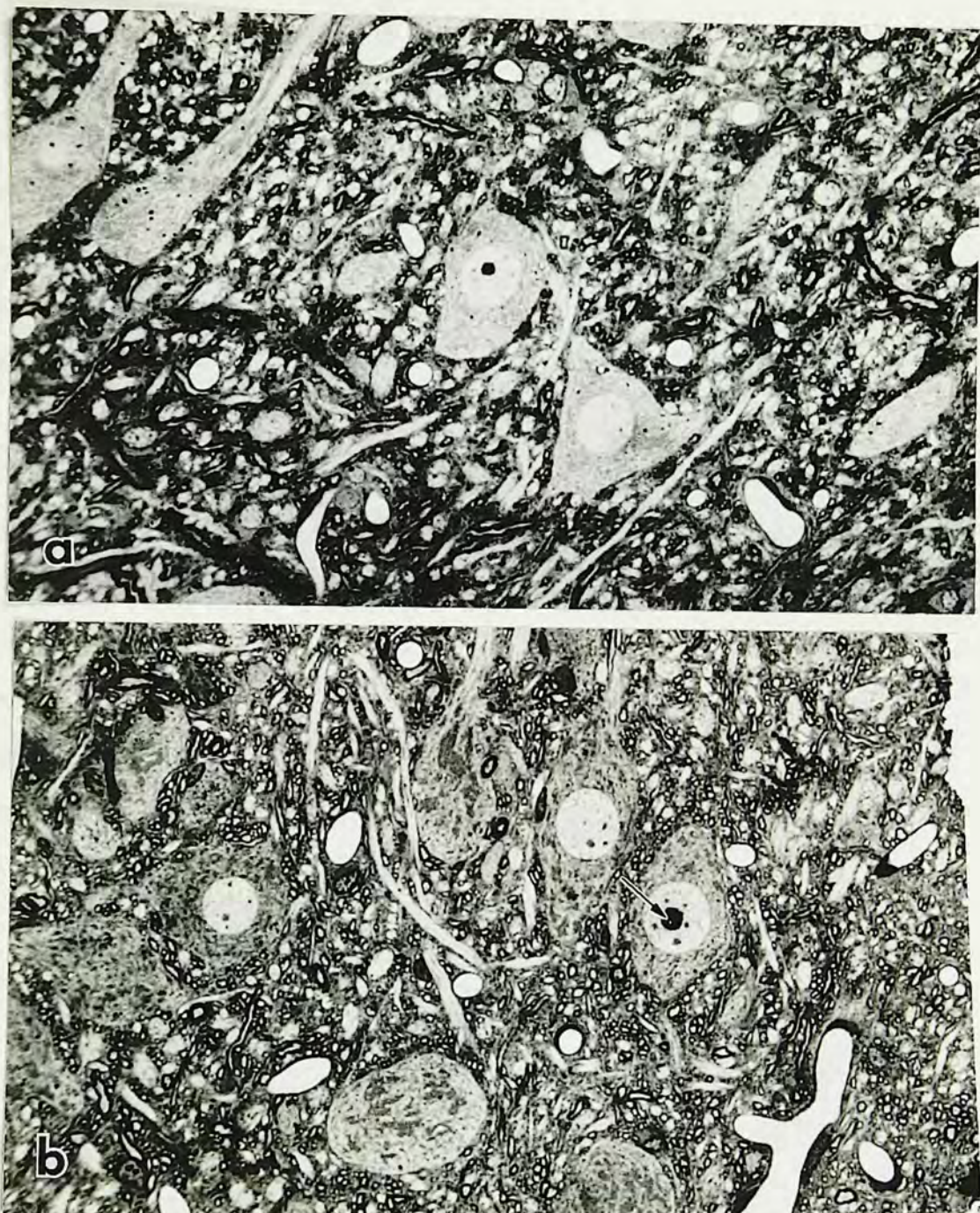


Figure 32

axotomy (Table 2). Large vacuoles were detected in the nucleoli of motoneurons 7 to 30 days following axotomy.

Electron Microscopic Observations

At the ultrastructural level, the uninjured motoneurons from hyperthyroid animals appeared identical to those from euthyroid animals (Fig 33). The axotomy induced ultrastructural changes exhibited by neurons from hyperthyroid animals generally resembled the retrograde response exhibited by the euthyroid population (Figs 34, 35). Some ultrastructural alterations provoked by axotomy among hyperthyroid animals differed from the euthyroid response. Among the hyperthyroid population, the nucleoli of axotomized motoneurons often contained greatly enlarged vacuoles 7 to 30 days following axotomy (Fig 36, 37a). In some axotomized neurons, multiple nucleoli were observed (Fig 37b). Also, a few axotomized motoneurons from the hyperthyroid animals contained nuclear filaments within the nucleus (Fig 37b).

Hypoglossal Nucleus Volume

The average volume of the control hypoglossal nuclei from hyperthyroid animals was $0.54 \pm 0.01 \text{ mm}^3$, a value similar to the control estimate from the euthyroid population (Fig 38). Hypoglossal volume was minimally decreased ($p > 0.05$) among the hyperthyroid group 7 to 21 days following axotomy (Fig 19b). By 30 days post-axotomy, a

Figure 33. An uninjured motoneuron from a hyperthyroid animal contains a spherical nucleus with a dense nucleolus (arrowhead). Nissl bodies are distributed throughout the perikarya (4,485X).

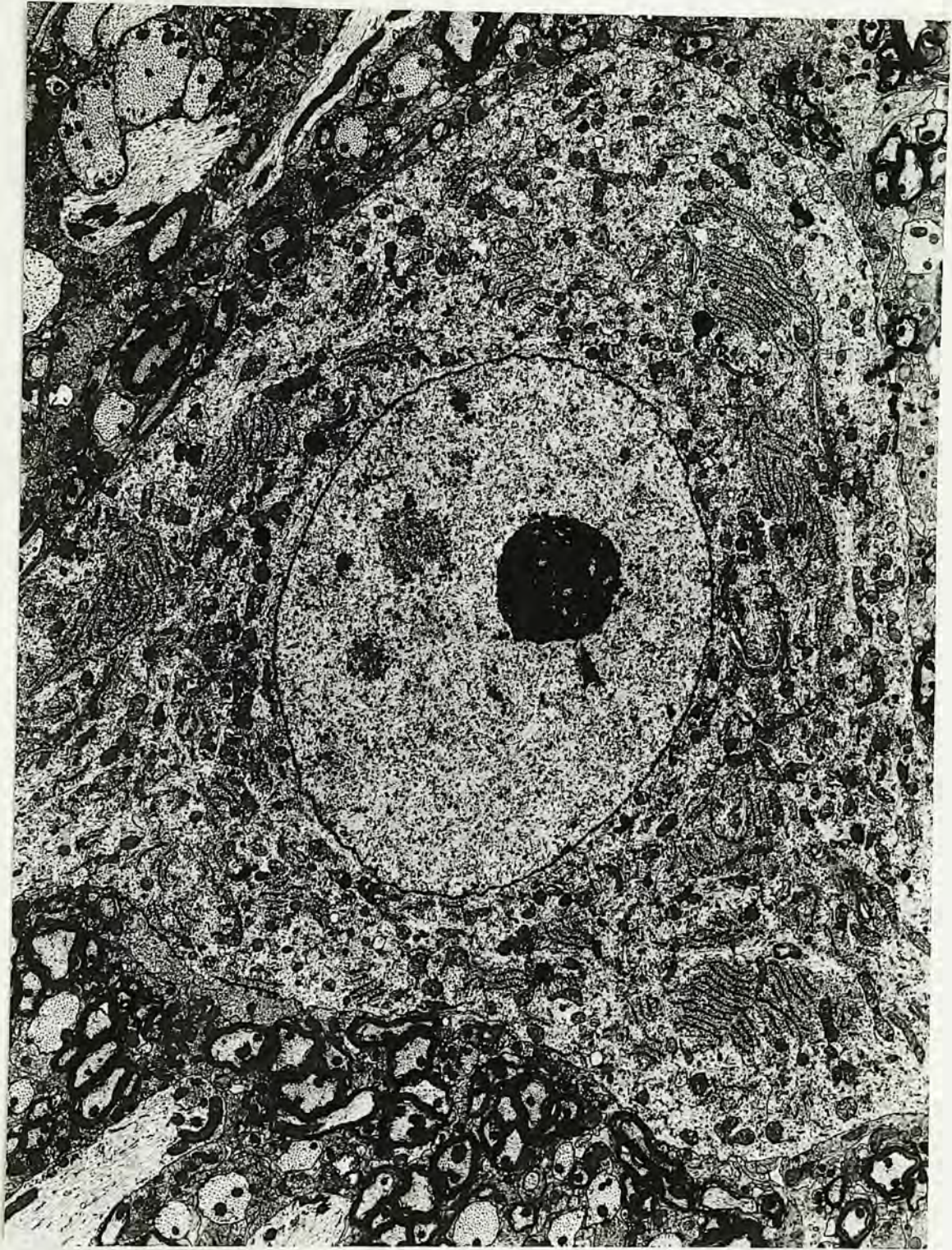


Figure 33

Figure 34. This is a motoneuron from a hyperthyroid animal, 7 days following axotomy. The nuclear envelope is invaginated and the "capping" cytoplasm is enriched with RER and polyribosomes (arrows). The nucleolus contains vacuoles. Modest disruption of the RER is apparent centrally (6,435X).

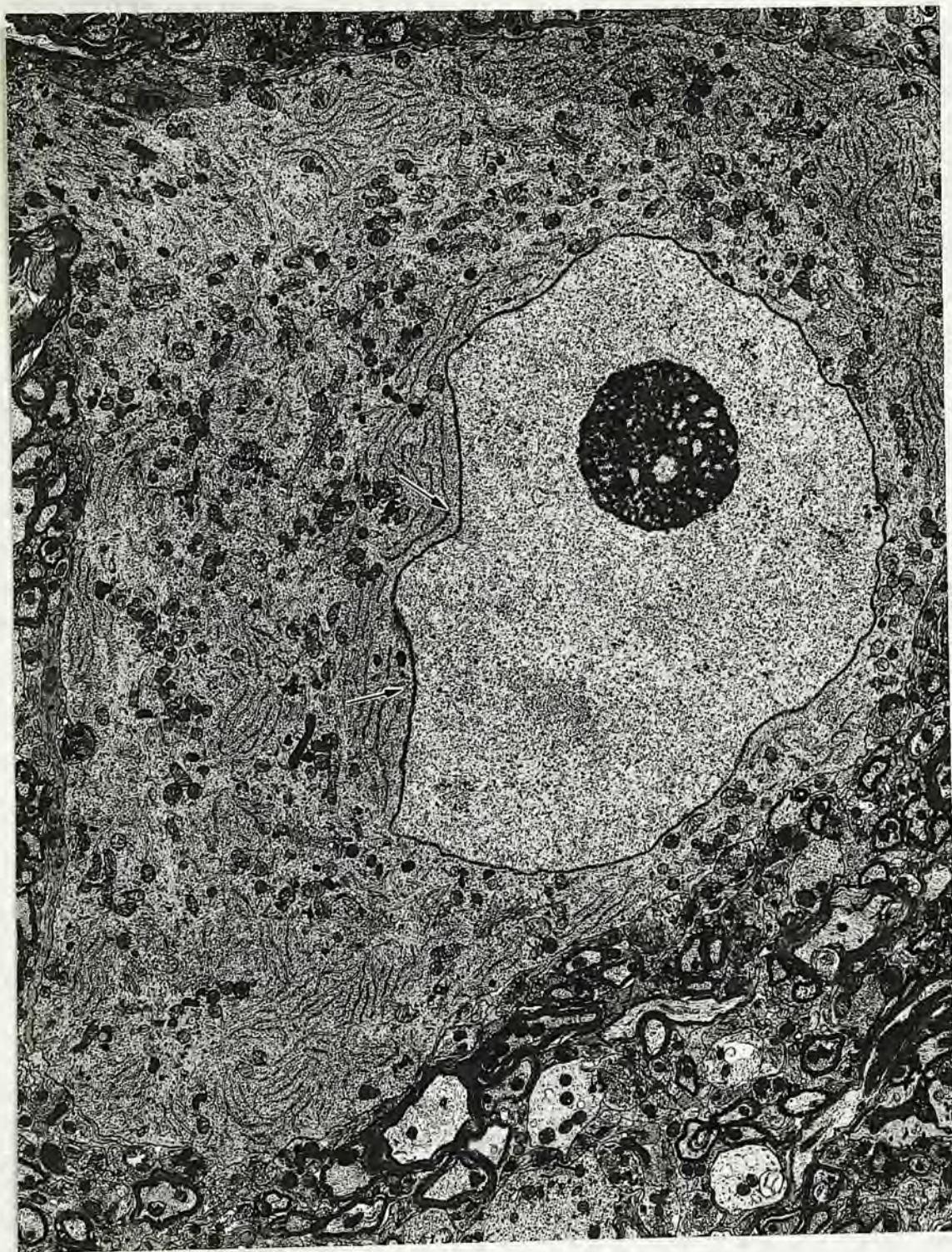


Figure 34

Figure 35. This motoneuron, from a hyperthyroid animal 13 days following axotomy, contains a spherical and centrally-located nucleus. The cisterns of RER are disorganized throughout the perikarya (6,630X).

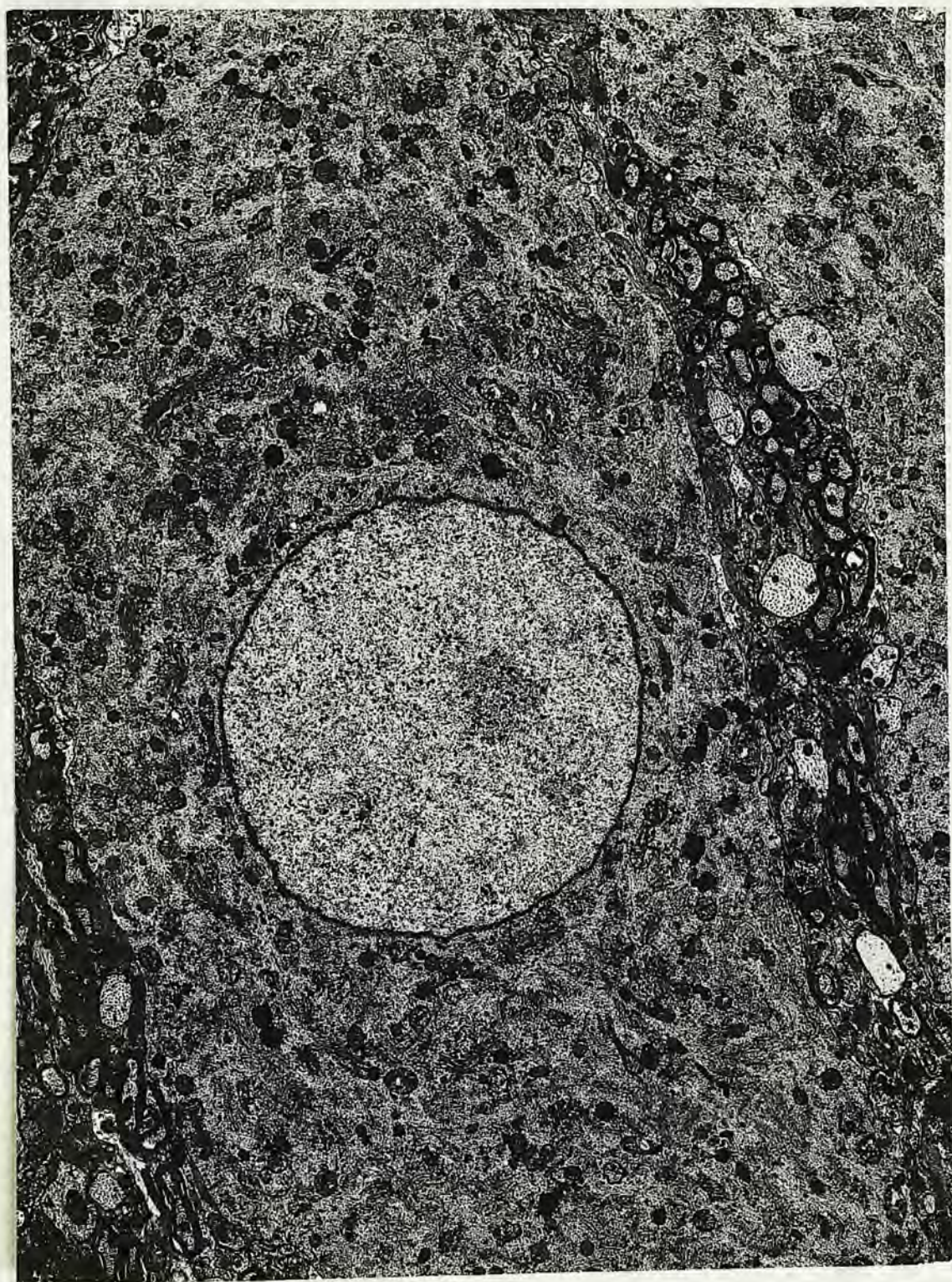


Figure 35

Figure 36. This nucleolus, from a motoneuron of a hyperthyroid animal 21 days following axotomy, contains several enlarged vacuoles (13,750X).

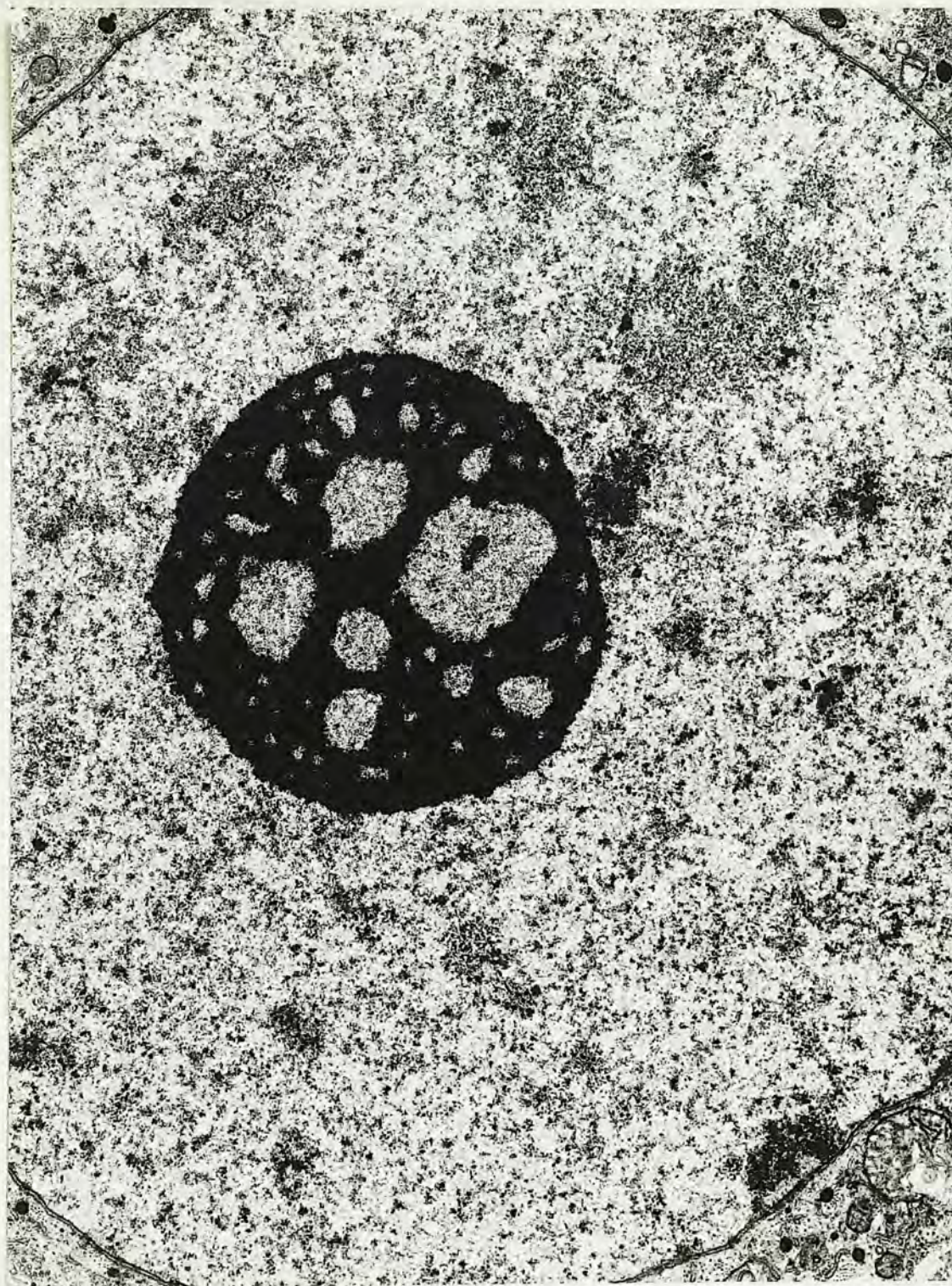


Figure 36

Figure 37a. Two nucleoli were observed in this motoneuron, from a hyperthyroid animal, 13 days following axotomy. One nucleolus (arrowhead) contains an enlarged vacuole (12,650X).

Figure 37b. In this motoneuron, taken from a hyperthyroid animal 30 days following axotomy, 3 nucleoli were present. A nuclear filament (arrowhead) was also observed within this neuron (9,350X).

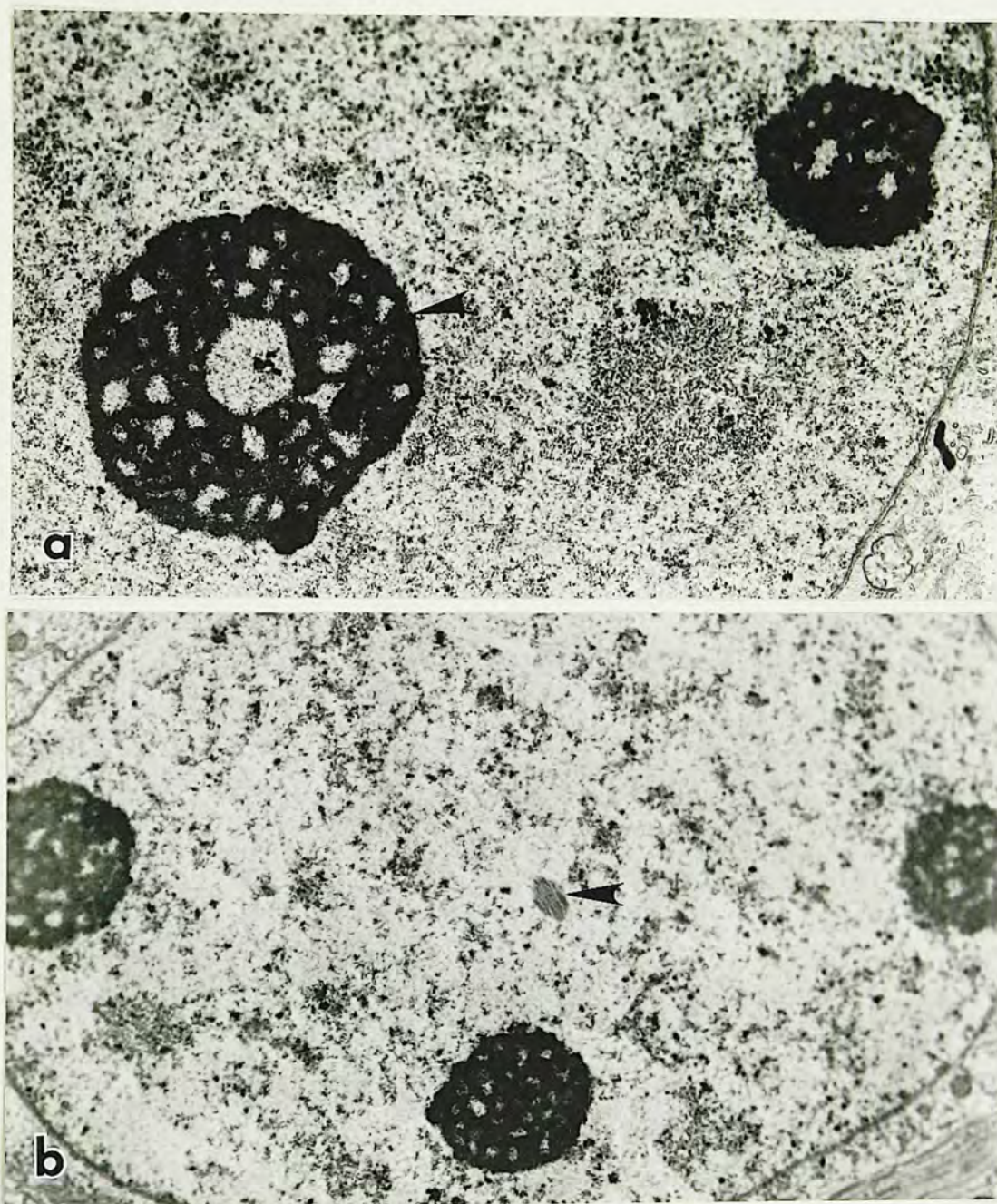


Figure 37

Figure 38. Mean volume (\pm SEM) of uninjured hypoglossal nuclei from the three thyroid hormone treatment groups. Different superscripts indicate significant differences among the groups ($p < 0.05$).

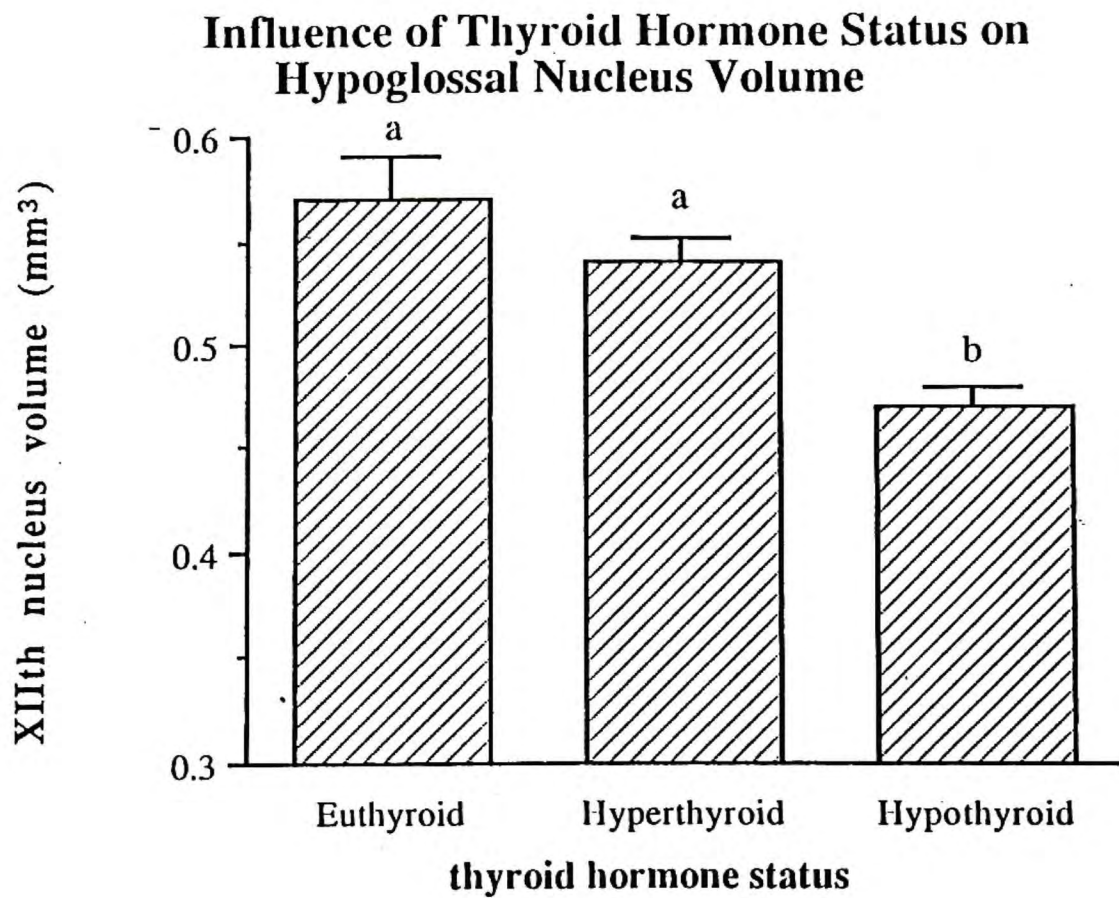


Figure 38

significant decrease ($p < 0.05$) in hypoglossal volume was observed among the hyperthyroid animals; the hypoglossal nucleus volume averaged $0.48 \pm 0.01 \text{ mm}^3$ at that time.

Neuronal Cell Body Size

All parameters reflecting neuronal cell body size evidenced neuronal enlargement following axotomy within the hyperthyroid group (Table 3). Neuronal fractional volume averaged $13.7 \pm 0.6\%$ among the uninjured hyperthyroid animals, a value similar to the estimate from the control euthyroid population. Within the hyperthyroid group, the neuronal fractional volume increased by approximately 20% from the measured control value ($p < 0.05$) 7 to 21 days post-axotomy. Neuronal fractional volume demonstrated a further increase 30 days following axonal transection among the hyperthyroid animals, averaging $20.3 \pm 0.3\%$ at this time ($p < 0.05$). Estimates of total neuronal volume, average neuronal cross-sectional area and average maximal diameter from hyperthyroid animals exhibited a response pattern similar to that depicted by neuronal fractional volume.

Volume and Surface Area of the Cell Nucleus

The neuronal nuclear volume and surface area were not significantly altered following axotomy among the hyperthyroid animals, as was observed within the euthyroid group. Nuclear fractional volume averaged $22.2 \pm 0.9\%$ among the hyperthyroid animals, which was not significantly

different from the corresponding euthyroid value (Table 4). Total nuclear volume, which slightly though not significantly increased following axotomy, averaged $0.018 \pm 0.002 \text{ mm}^3$ among the hyperthyroid animals. Nuclear surface density and surface area averaged $1,021.9 \pm 38.7/\text{cm}$ and $8.6 \pm 0.3 \text{ mm}^2$ respectively, among the hyperthyroid animals; nuclear surface area exhibited a modest, but non-significant increase following axotomy (Fig 20b).

Nucleolar Volume

Nucleolar volume, fractional and total, was not significantly altered following axotomy among the hyperthyroid animals (Fig 21b, 22b). Average nucleolar fractional volume was $3.00 \pm 0.30\%$ among all hyperthyroid animals and total nucleolar volume averaged $5.68 \pm 0.85 \times 10^5 \text{ um}^3$. The fractional and total nucleolar volumes measured among hyperthyroid animals were slightly larger than those observed among the hypothyroid and uninjured euthyroid populations.

THE RETROGRADE RESPONSE AMONG THE HYPOTHYROID POPULATION

Light Microscopic Observations of Vibratome Sections

Features of the uninjured hypoglossal nuclei from hypothyroid animals, revealed in 50 micron sections, were analogous to those exhibited by the euthyroid population (Fig 39a). Also, the retrograde response to axotomy

Figure 39a. The uninjured hypoglossal nuclei (outlined), pictured in this 50 micron coronal section through the medulla of a hypothyroid animal, contain densely stained neurons and a reticulated neuropil replete with neurites, glia and blood vessels. The left and right hypoglossal nuclei have similar cross-sectional profiles (48X).

Figure 39b. Thirteen days following axotomy, the cross-sectional profile of the injured hypoglossal nucleus appears somewhat smaller than the contralateral side in this 50 micron section from the medulla of a hypothyroid rat (48X).

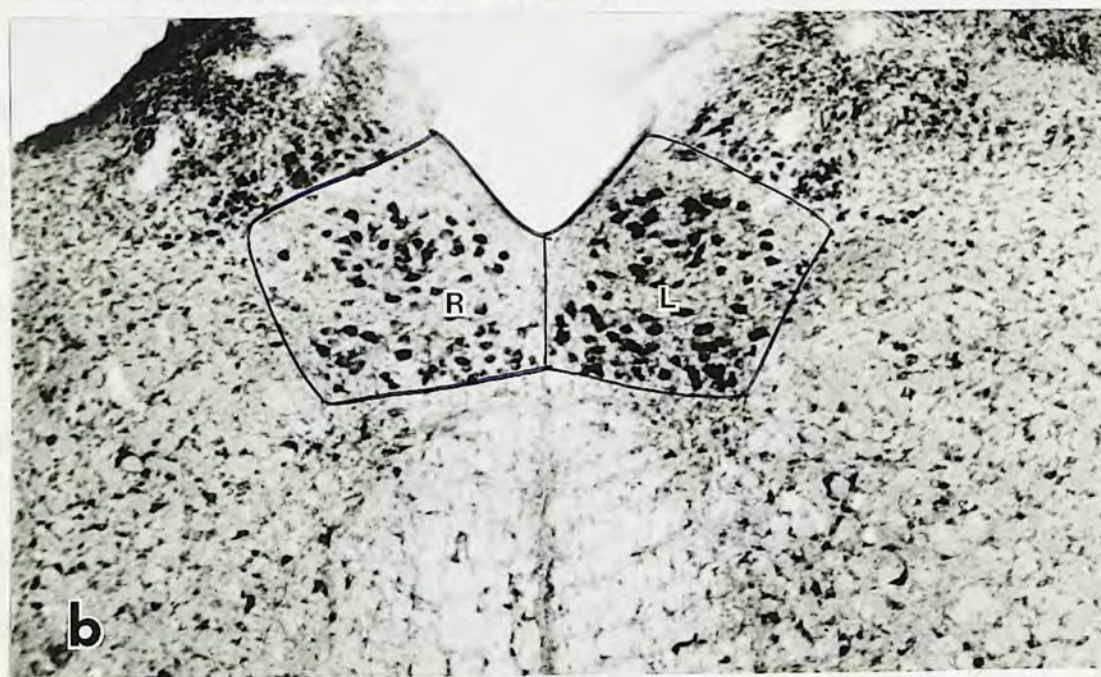


Figure 39

generally appeared similar among the hypothyroid and euthyroid population, in 50 micron sections (Fig 39b). The hypothyroid animals displayed such characteristic retrograde responses to axotomy as diminished hypoglossal nucleus cross-sectional profile area, increased neuronal basophilia and glial proliferation.

Light Microscopic Observations of Plastic Embedded Sections

The uninjured motoneurons and surrounding neuropil from the hypoglossal nuclei of hypothyroid animals were indistinguishable from the corresponding euthyroid population when viewed in one micron sections (Fig 40a). Many features of the retrograde response to axotomy exhibited by motoneurons from hypothyroid animals paralleled the euthyroid response (Fig 40b). However, among the hypothyroid population, cell nucleus eccentricity and "ruffling" of the nuclear envelope were expressed by 90% of the motoneurons examined 7 days following the nerve lesion (Table 2).

Electron Microscopic Observations

The ultrastructure of the uninjured motoneurons from hypothyroid animals appeared identical to the euthyroid counterpart. Axotomy provoked many of the same ultrastructural changes within neurons from hypothyroid animals as reported among the euthyroid population (Fig 41, 42, 43). The appearance of nuclear filaments within the

Figure 40a. The uninjured motoneurons from the hypothyroid animals, as seen in this one micron section from the hypoglossal nucleus, contained spherical nuclei, densely stained nucleoli, and basophilic Nissl bodies (960X).

Figure 40b. Seven days following axotomy, as shown in this one micron section from the hypoglossal nucleus of a hypothyroid animals, many neurons contained deformed nuclei (960X).

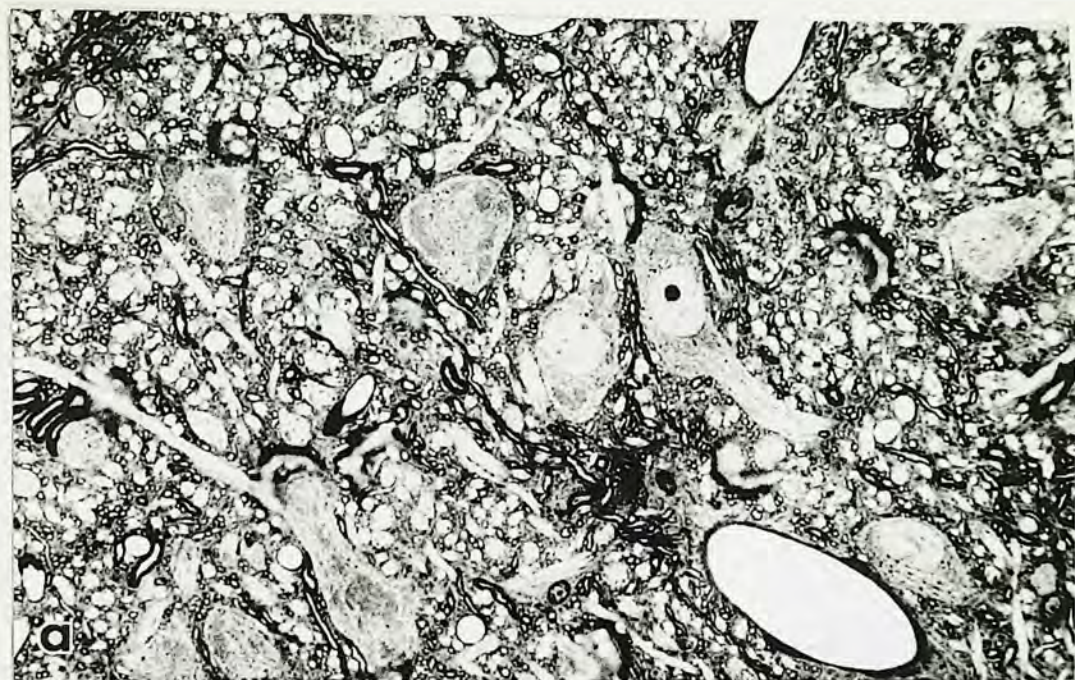


Figure 40

Figure 41. Modest invagination of the nuclear envelope and "capping" with RER and polyribosomes (arrow) were observed in this motoneuron, from a hypothyroid animal 7 days following axotomy (3,978X).

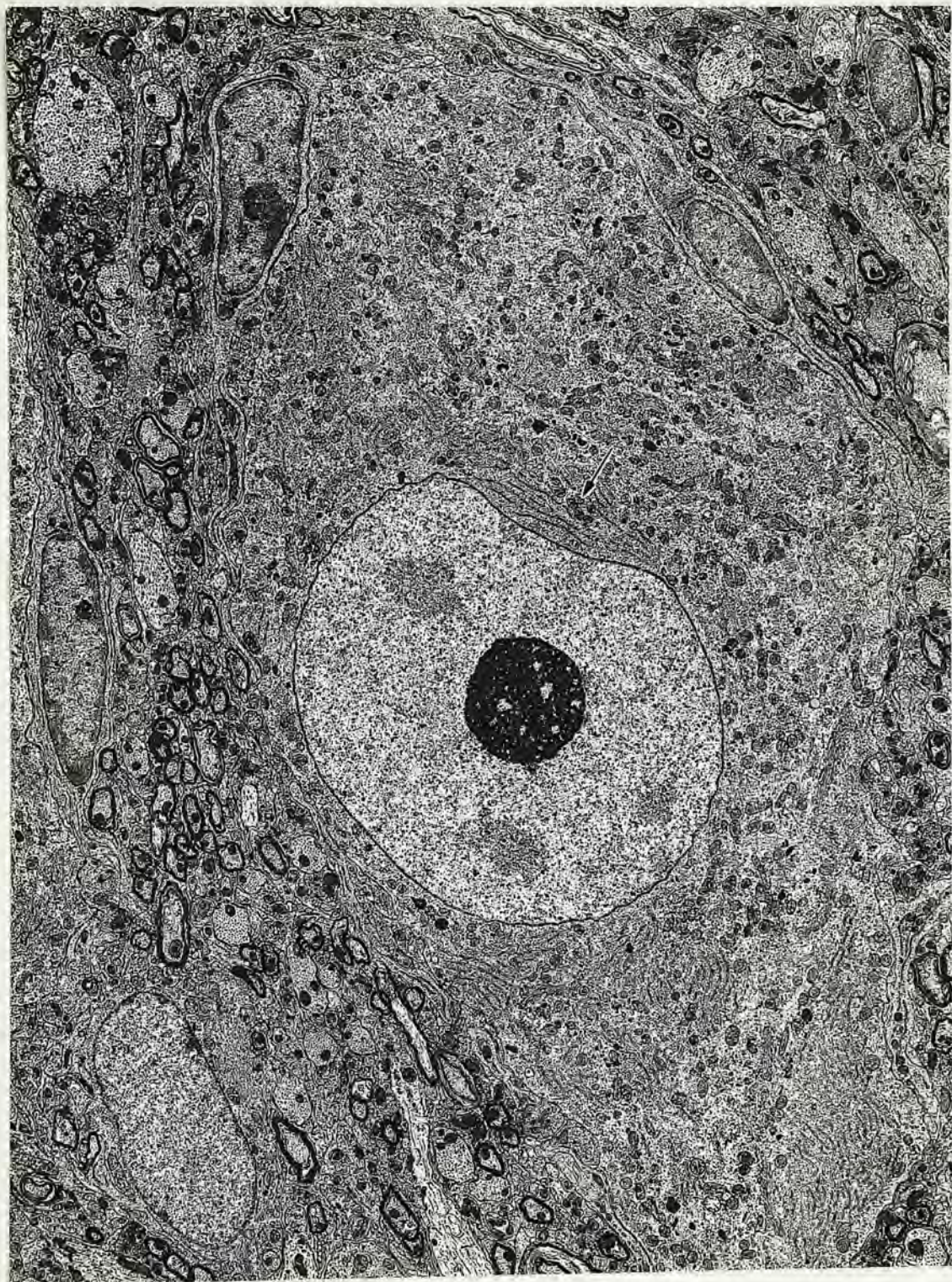


Figure 41

Figure 42. This motoneuron, from a hypothyroid animal 7 days post-axotomy, contained an evaginating nucleus, which is sectioned into 2 profiles (arrowheads). The cytoplasm near the nucleus is filled with polyribosomes and RER. Chromatolysis is evident in other regions of the perikaryon (6,500X).

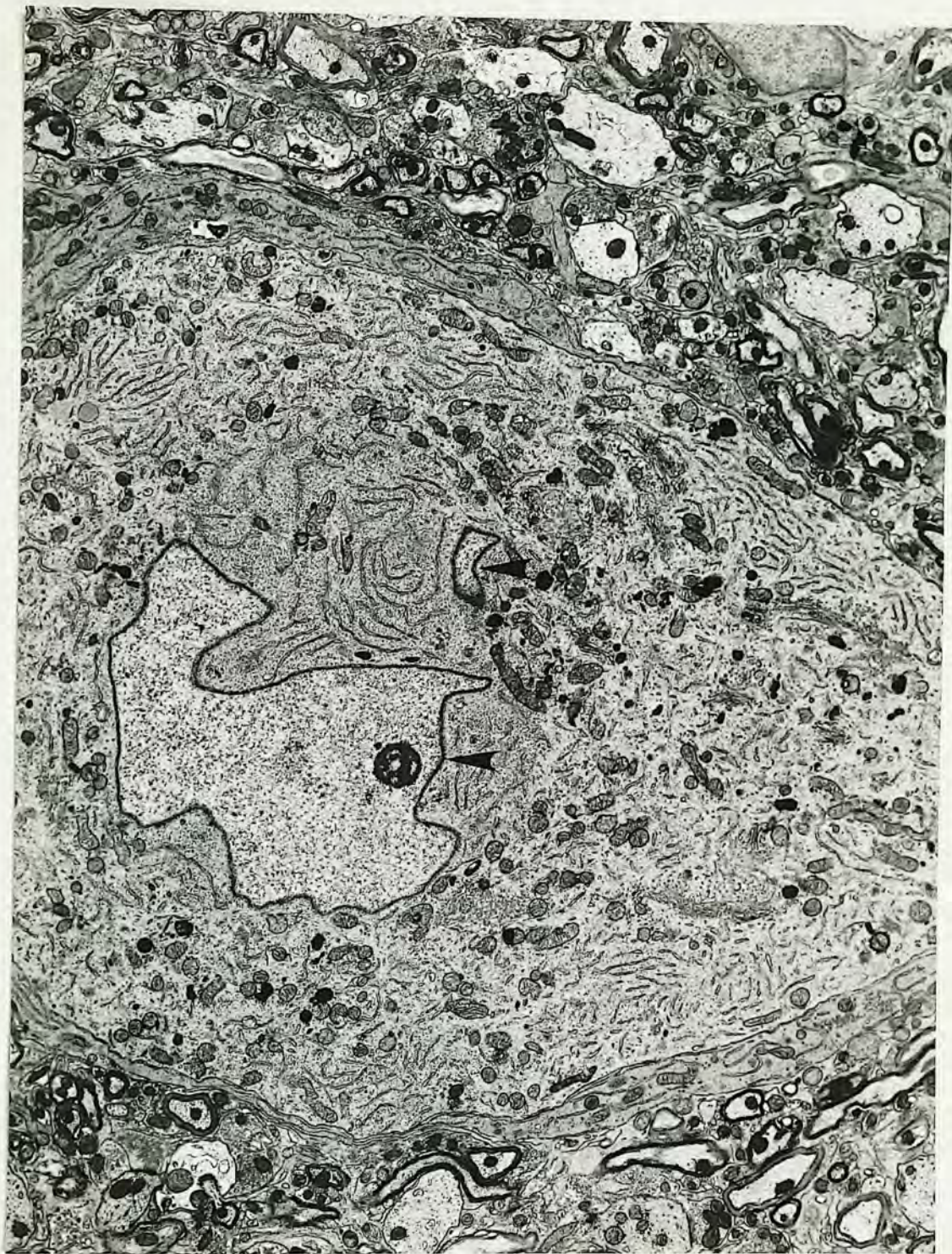


Figure 42

Figure 43. This nucleus was observed within a motoneuron, from a hypothyroid animal, 7 days following axotomy. The nuclear envelope is invaginated. Small vacuoles can be seen pervading the nucleolus (arrow) (10,750X).

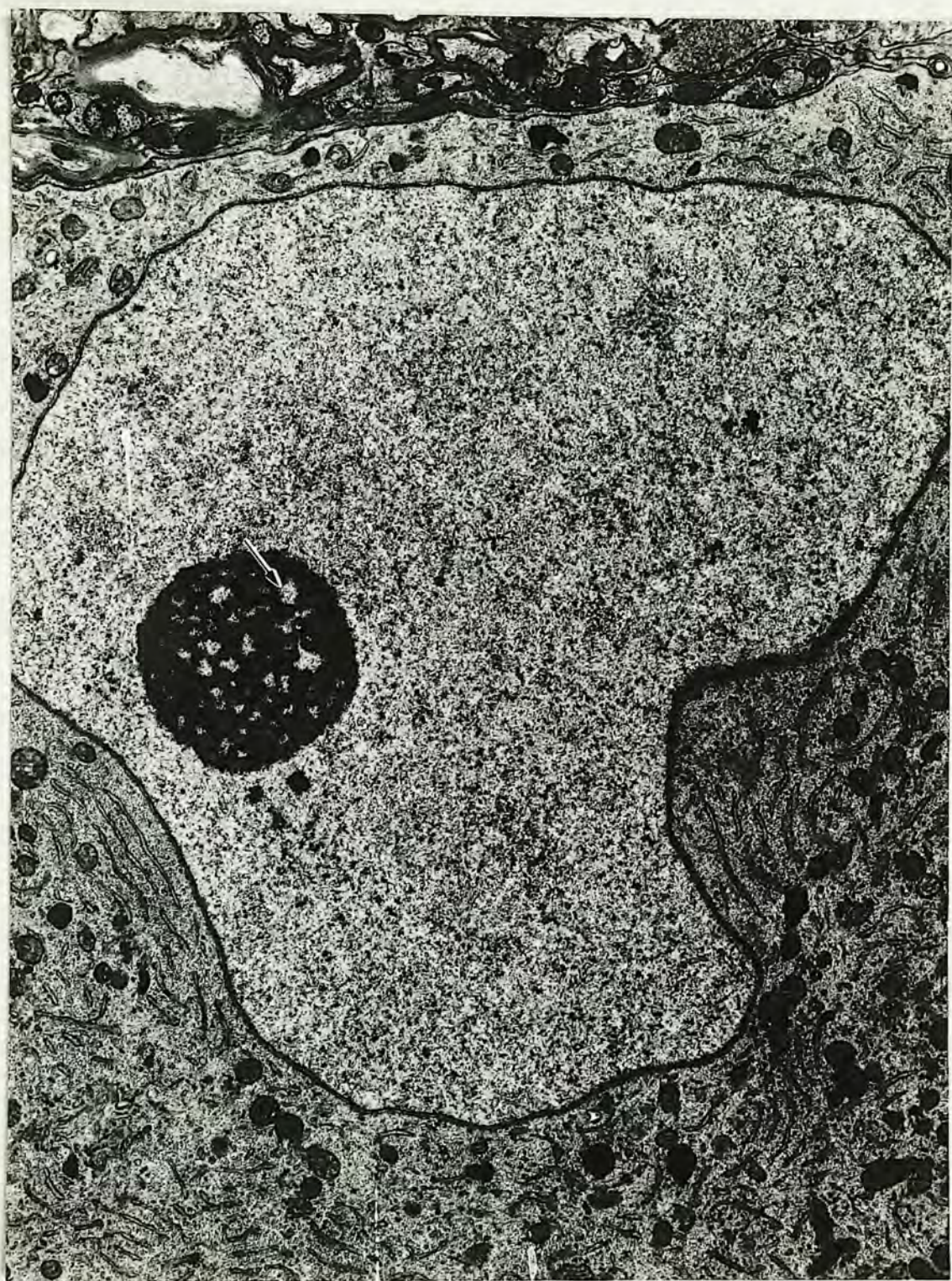


Figure 43

nuclei of axotomized motoneurons distinguished the ultrastructural retrograde response of hypothyroid animals from the euthyroid group (Fig 44 a,b).

Hypoglossal Nucleus Volume

The average volume of uninjured hypoglossal nuclei from hypothyroid animals was $0.47 \pm 0.01 \text{ mm}^3$ (Fig 38). This value is significantly less than the average from uninjured hypoglossal nuclei of eu- and hyperthyroid animals ($p < 0.05$). Hypoglossal volume demonstrated a further significant diminution among the hypothyroid rats ($p < 0.05$) 7 to 30 days following axotomy (Fig 19c).

Neuronal Cell Body Size

All estimates of neuronal size derived from uninjured hypoglossal nuclei of hypothyroid animals were similar to the corresponding estimates from the eu- and hyperthyroid populations (Table 3). Among the hypothyroid animals, neuronal fractional volume significantly exceeded the control average 7 to 30 days following axotomy ($p < 0.05$). All other parameters estimating neuronal size failed to demonstrate axotomy-induced neuronal enlargement among the hypothyroid animals. Total neuronal volume averaged $0.070 \pm 0.006 \text{ mm}^3$ among all hypothyroid rats and the average cross-sectional area and maximal diameter were $529.3 \pm 20.2 \text{ um}^2$ and $32.6 \pm 0.8 \text{ um}$, respectively.

Figure 44a. A nucleolus (arrow) and nuclear filament (arrowhead) were observed within this motoneuron from a hypothyroid animal 21 days following axotomy (42,500X).

Figure 44b. Within this motoneuron, from a hypothyroid animal 13 days post-axotomy, a small nucleolus is located near the inner lamina of the nuclear membrane. A nuclear filament (arrowhead) is near the nucleolus (23,400X).

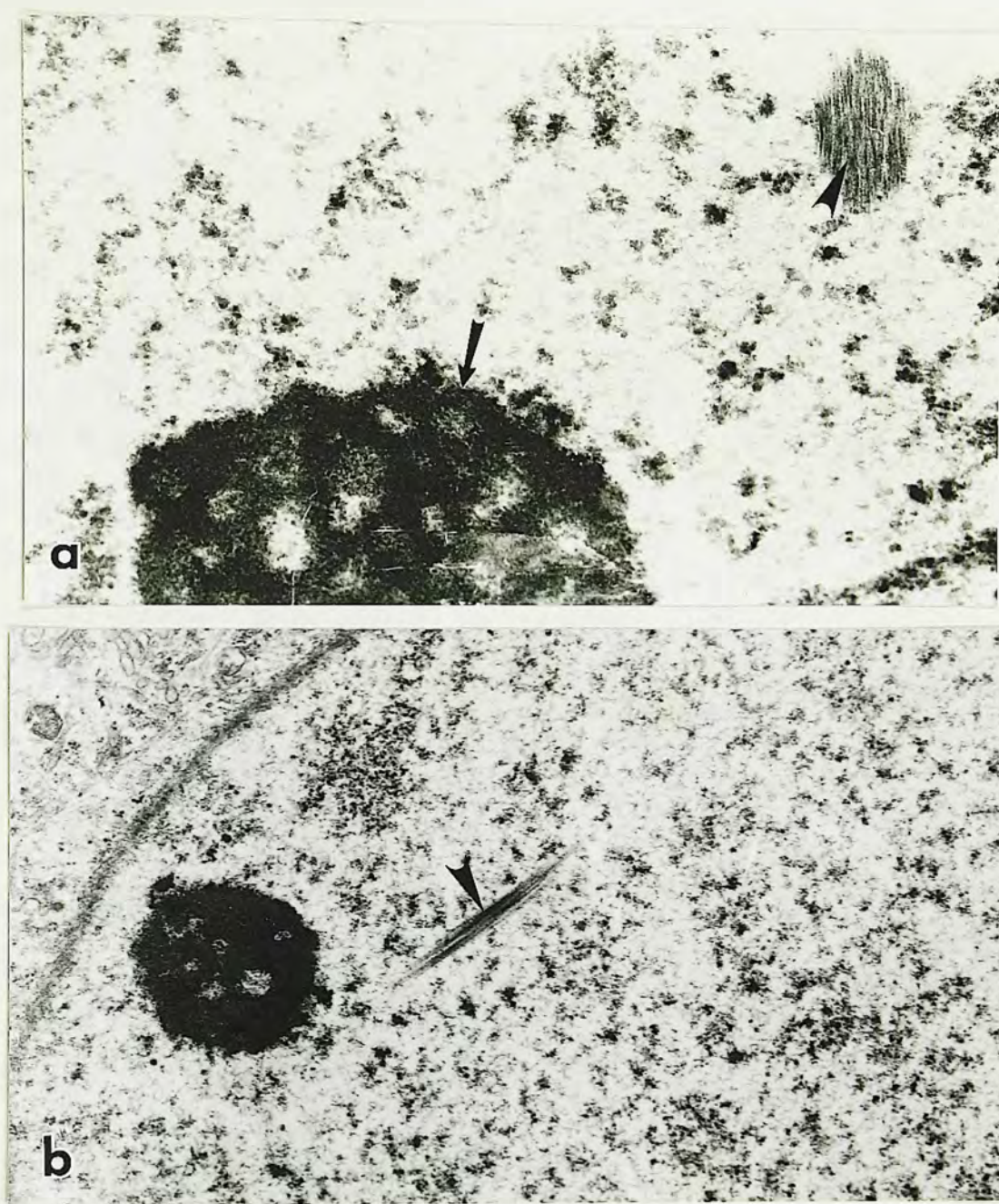


Figure 44

Volume and Surface Area of the Cell Nucleus

As observed among the eu- and hyperthyroid animals, cell nucleus fractional and total volume were constant following axotomy among the hypothyroid population (Table 4). Nuclear fractional and total volume averaged $23.6 \pm 0.7\%$ and $0.017 \pm 0.001 \text{ mm}^3$, respectively. These measurements are similar to corresponding estimates from the eu- and hyperthyroid animals. Nuclear surface density averaged $1,099.3 \pm 39.7/\text{cm}$ among control hypothyroid animals, comparable to corresponding eu- and hyperthyroid values. A significant increase in nuclear surface density and area was observed among the hypothyroid population 7 days following axotomy (Fig 20C). Total nuclear surface area was also significantly influenced by axotomy ($p < 0.05$). Among uninjured neurons from the hypothyroid animals, total nuclear surface area averaged $7.5 \pm 0.5 \text{ mm}^2$, a value not significantly different from the control eu- and hyperthyroid population averages. Among the hypothyroid animals, the total nuclear surface area surpassed the control value by 28%, 7 days following axotomy (Fig 20C). This transitory increase in nuclear surface area had discontinued 13 to 30 days following axotomy.

Nucleolar Volume

The fractional and total nucleolar volume proved to be constant following axotomy among the hypothyroid animals (Fig 21C, 22C). The overall average nucleolar fractional

(Fig 21C, 22C). The overall average nucleolar fractional volume was $2.22 \pm 0.14\%$ among the hypothyroid population. Total nucleolar volume averaged $3.72 \pm 0.45 \times 10^5 \text{ um}^3$ among the hypothyroid group. These values did not significantly differ from those observed among the corresponding hyperthyroid population and uninjured euthyroid population.

THYROID HORMONE STATUS AND NERVE REGENERATION

Labeling of Hypoglossal Motoneurons from the Euthyroid Population with Horseradish Peroxidase

Among the uninjured euthyroid population, 64% of the hypoglossal motoneurons analyzed were labeled with tongue-injected HRP (Fig 45). Among unoperated animals, the number of HRP labeled neurons in the left and contralateral, right hypoglossal nuclei were similar.

HRP was not detected in any neuronal cell body 13 days following axotomy (Table 5). Eighteen days following axotomy, the percentage of neurons containing HRP reaction product averaged 43% among the euthyroid animals (Fig 46). From 21 to 50 days following nerve lesion, a progressive increase of neuronal HRP labeling was observed among euthyroid animals; the percentage of HRP-positive neurons ranged from 41 to 54% during this interval. The ratio of HRP positive neurons in the injured versus uninjured, contralateral hypoglossal nucleus ranged from 25 to 75% during this interval. Following axotomy, the total number

Figure 45. Ratio of uninjured neurons identified as HRP-positive/total number of neurons counted. Means (\pm SEM) are reported. Different superscripts denote significant differences among means.

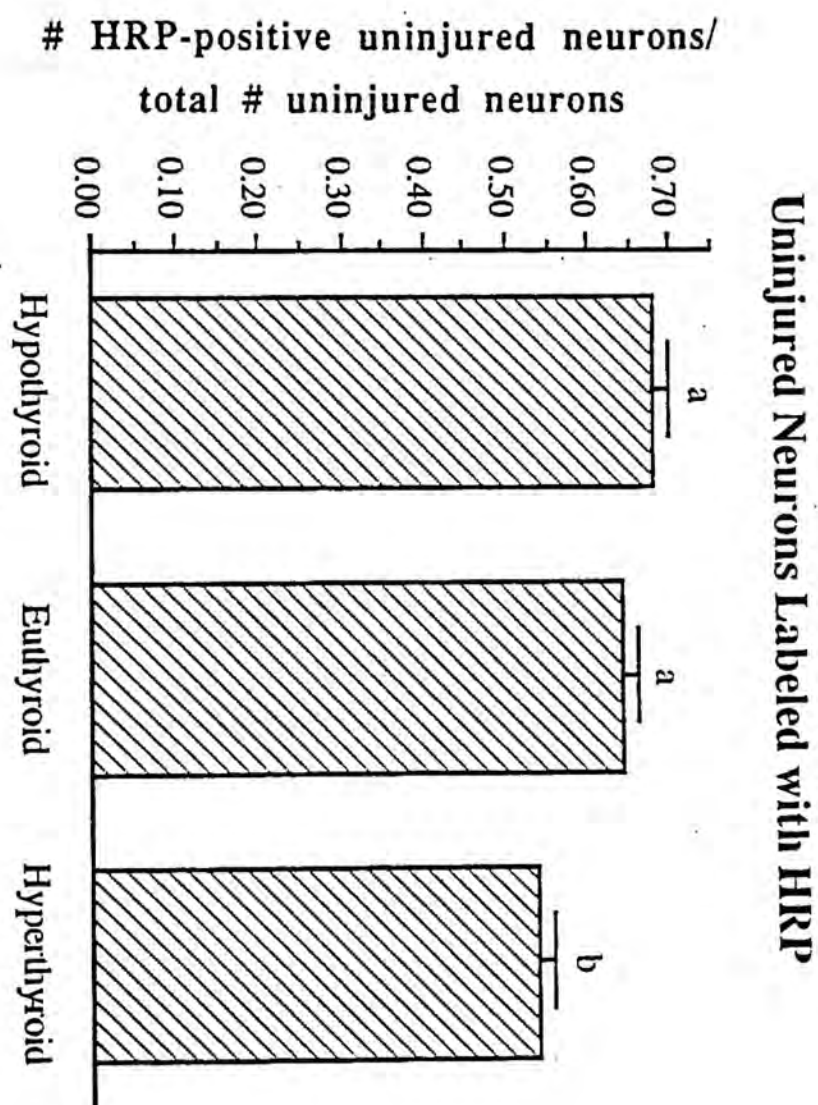


Figure 45

TABLE 5.

AXONAL REGENERATION AND FUNCTIONAL RECOVERY

	Days Following Axotomy ^a					
	13	18	21	30	40	50
Euthyroid						
HRP labeled Neurons ^b	0	+	+	+	+	+
Functional Recovery ^c	0	0	+	+	+	+
Hyperthyroid						
HRP labeled Neurons	0	+	+	+	+	+
Functional Recovery	0	0	+	+	+	+
Hypothyroid						
HRP labeled Neurons	0	0	+	+	+	+
Functional Recovery	0	0	0	+	+	+

^aEach symbol represents 2 to 4 animals.

^b0 indicates that HRP was not detected in the hypoglossal nucleus 36 hours following injection into the tongue; + indicates that hypoglossal motoneurons with HRP were detected following tongue injection.

^c0 indicates that tongue muscle force was not detected following stimulation of the axotomized hypoglossal nerve; + indicates that tongue muscle force was detected following stimulation of the axotomized hypoglossal nerve.

Figure 46. Ratio of HRP-positive neurons/total number of neurons counted among eu-, hyper- and hypothyroid animals following axotomy. Each point represents at least 400 total neurons counted. Chi-square analysis revealed that the proportions varied significantly among the groups ($p < 0.005$). The value from hypothyroid animals, 18 days following axotomy, contributed more than 40% of the chi-square value.

The Influence of Thyroid Hormone Status on HRP Labeling

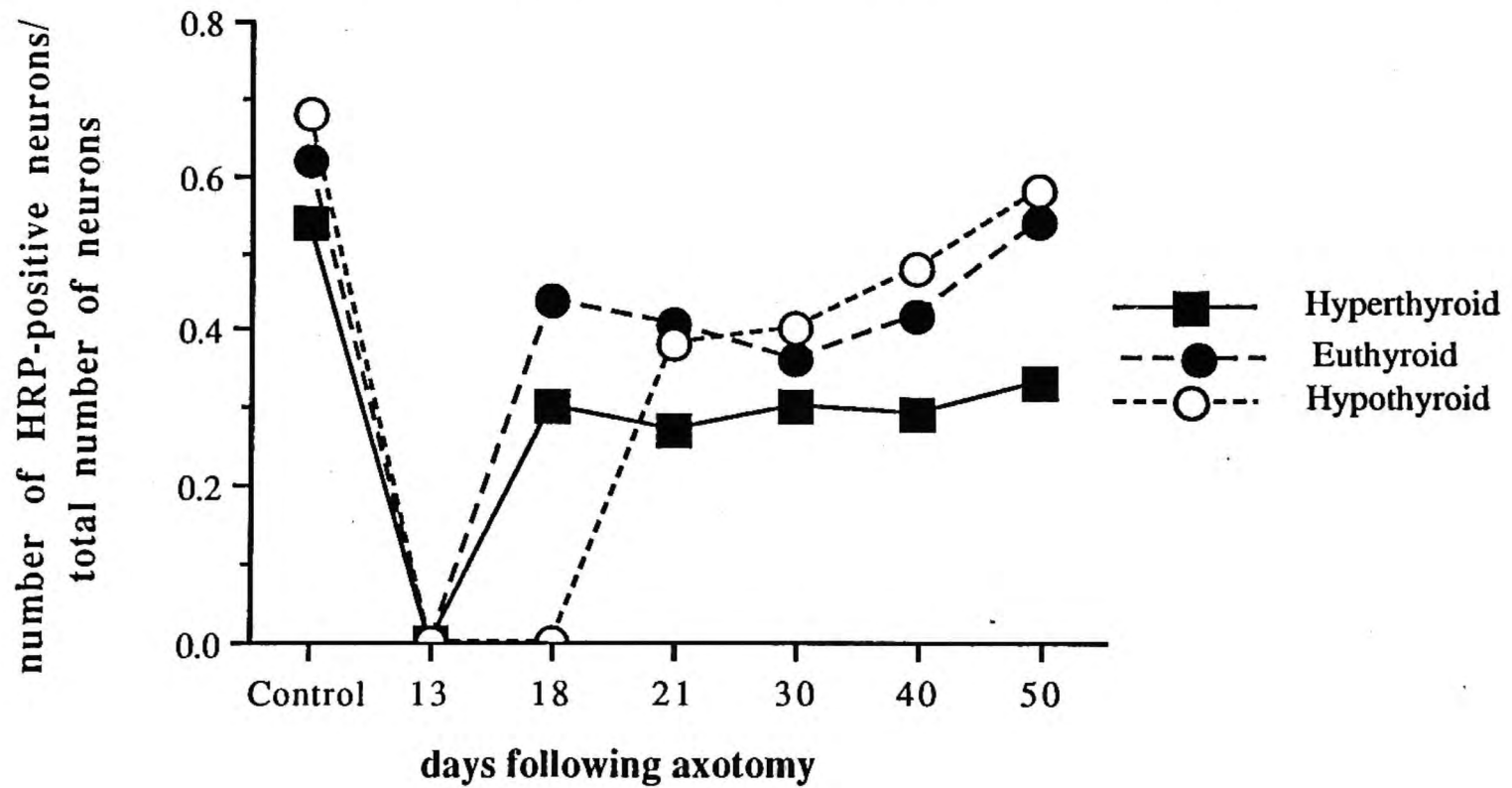


Figure 46

of motoneurons counted in the injured and uninjured hypoglossal nuclei did not significantly vary at any post-axotomy interval analyzed (Fig 47).

Labeling of Hypoglossal Motoneurons from the Hyperthyroid Population with Horseradish Peroxidase

HRP labeling of uninjured motoneurons was significantly less among the hyperthyroid animals, than among the eu- and hypothyroid populations ($p < 0.05$), averaging 51% (Fig 45). HRP labeling of the right and contralateral left hypoglossal nuclei, among unoperated, hyperthyroid animals, did not differ significantly.

Retrograde transport of HRP to the neuronal cell bodies was not apparent 13 days following axotomy among the hyperthyroid population (Table 5). Neurons containing HRP were first observed among the hyperthyroid population 18 days following the nerve lesion. The percentage of neurons containing HRP reaction product was constant 21 to 50 days following axotomy, averaging 30% (Fig 46). The percentage of HRP positive neurons in the injured versus uninjured, contralateral hypoglossal nucleus averaged 51% during this interval. Neuronal cell loss was not observed within axotomized hypoglossal nuclei from hyperthyroid animals 13 to 50 days following the nerve lesion (Fig 47).

Figure 47. Number of cells counted in injured/uninjured hypoglossal nucleus within the same 50 micron section. Each point represents at least 800 total neurons counted among the eu-, hyper- and hypothyroid animals. Chi-square analysis of the number of neurons counted revealed that, within each treatment group, the relative number of cells are the same in uninjured and injured hypoglossal nuclei 0 to 50 days following axotomy.

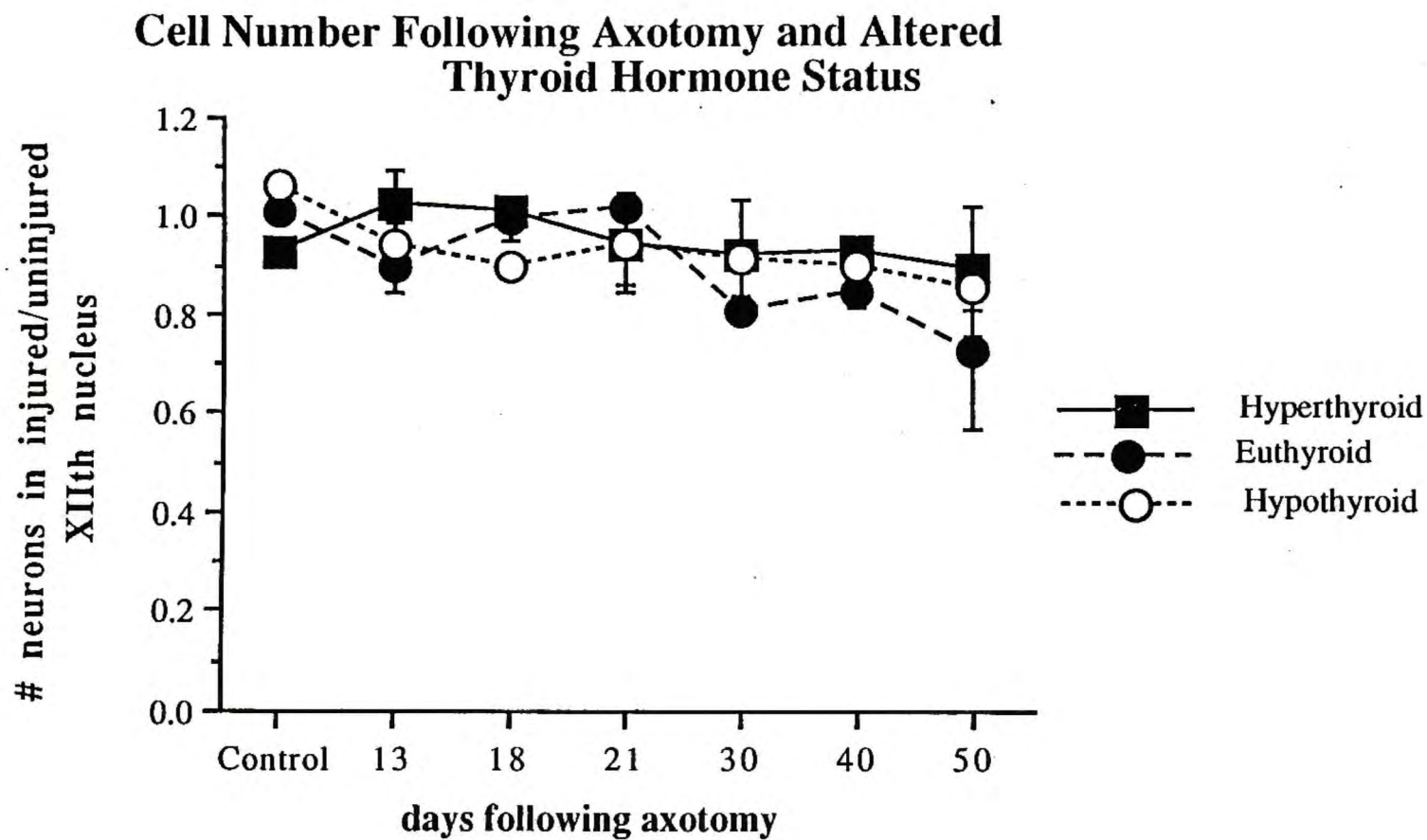


Figure 47

Labeling of Hypoglossal Motoneurons from the Hypothyroid Population with Horseradish Peroxidase

Sixty-seven percent of the motoneurons from uninjured hypothyroid animals contained HRP reaction product (Fig 45). HRP labeling of neurons in the left and contralateral right hypoglossal nuclei was similar among the unoperated, hypothyroid animals.

HRP labeling of axotomized motoneurons was not observed until 21 days following the nerve lesion among the hypothyroid population (Table 5). From 21 to 50 days following axotomy, a progressive increase of neuronal HRP labeling was observed, ranging from 39% to 57% during that interval (Fig 46). HRP labeling within the injured versus uninjured contralateral hypoglossal nuclei ranged from 27% to 79%, 21 to 50 days following axotomy. Among the hypothyroid animals, the number of neurons within the injured and uninjured hypoglossal nuclei were unaltered at all times evaluated following axotomy.

Hypoglossal Nerve Function among the Euthyroid Population

Axotomy resulted in denervation of the tongue as evidenced by the absence of stimulated tongue contraction 13 days following axonal transection (Table 5). Hypoglossal nerve functional recovery, defined as nerve stimulated tongue force, was first observed 21 days following axotomy among the euthyroid group (Table 5). HRP labeling of the neuronal cell bodies preceded the functional recovery.

Stimulated tongue force was observed in all euthyroid animals 30, 40 and 50 days following axotomy.

As depicted in Figure 48, increasing stimulating voltages evoked incremental increases in measured tongue force until a maximal value was obtained, beyond which no greater twitch force could be evoked. Stimulation of uninjured hypoglossal nerves provoked a maximal force of 10.4g among the euthyroid animals (Fig 49).

Functional reinnervation, first observed 21 days following axotomy, was characterized by a progressive increase in stimulated maximal force 21 to 50 days following the injury (Fig 50A). Even 50 days following axotomy, however, maximal force production was less than measured on the contralateral, uninjured side ($p < 0.01$). The recovery of maximal force production, expressed as the ratio of maximal force provoked following stimulation of the regenerated versus uninjured nerve, ranged from 0.10 at 21 days post-axotomy to 0.45, 50 days post-axotomy, among the euthyroid population.

Hypoglossal Nerve Function among the Hyperthyroid Population

Nerve stimulation failed to provoke tongue force production 13 days following axotomy among the hyperthyroid population (Table 5). The functional recovery of the hypoglossal nerve was first observed 21 days following axotomy among the hyperthyroid group, similar to the euthyroid response (Table 5). Among all hyperthyroid

Figure 48. Twitch muscle force measured in the tongue over a series of increasing stimuli voltages among euthyroid animals. Each point represents an average from two animals, three measurements/animal.

Force Produced by Stimulation of Uninjured and Regenerated Hypoglossal Nerves in Euthyroid Animals

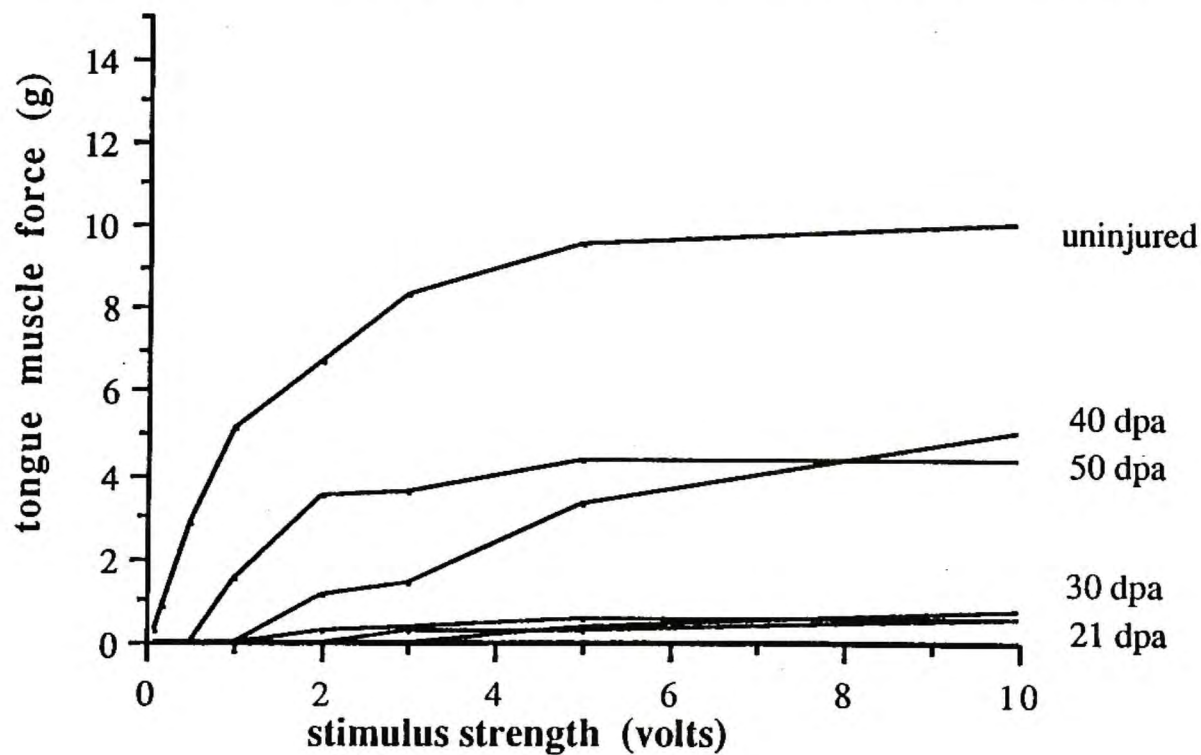


Figure 48

Figure 49. The maximal tongue force produced following stimulation of uninjured hypoglossal nerves from eu-, hyper- and hypothyroid animals is plotted. Different superscripts indicate significant differences ($p < 0.01$).

**Maximal Tongue Force Among Uninjured
Eu-, Hyper- and Hypothyroid Rats**

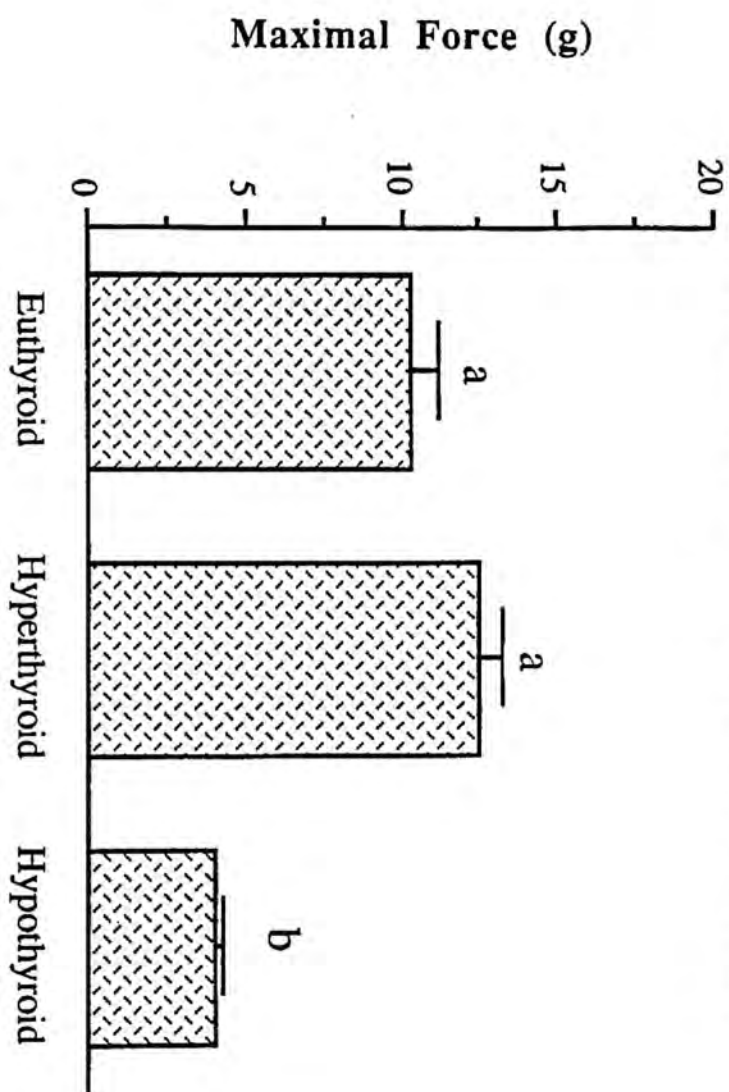


Figure 49

Figure 50. Maximal twitch force measured in the tongue following stimulation of the injured (I) and contralateral uninjured (U) nerve among eu- (a), hyper- (b) and hypothyroid (c) animals. Each bar is an average from two animals and three measurements/animal/nerve.

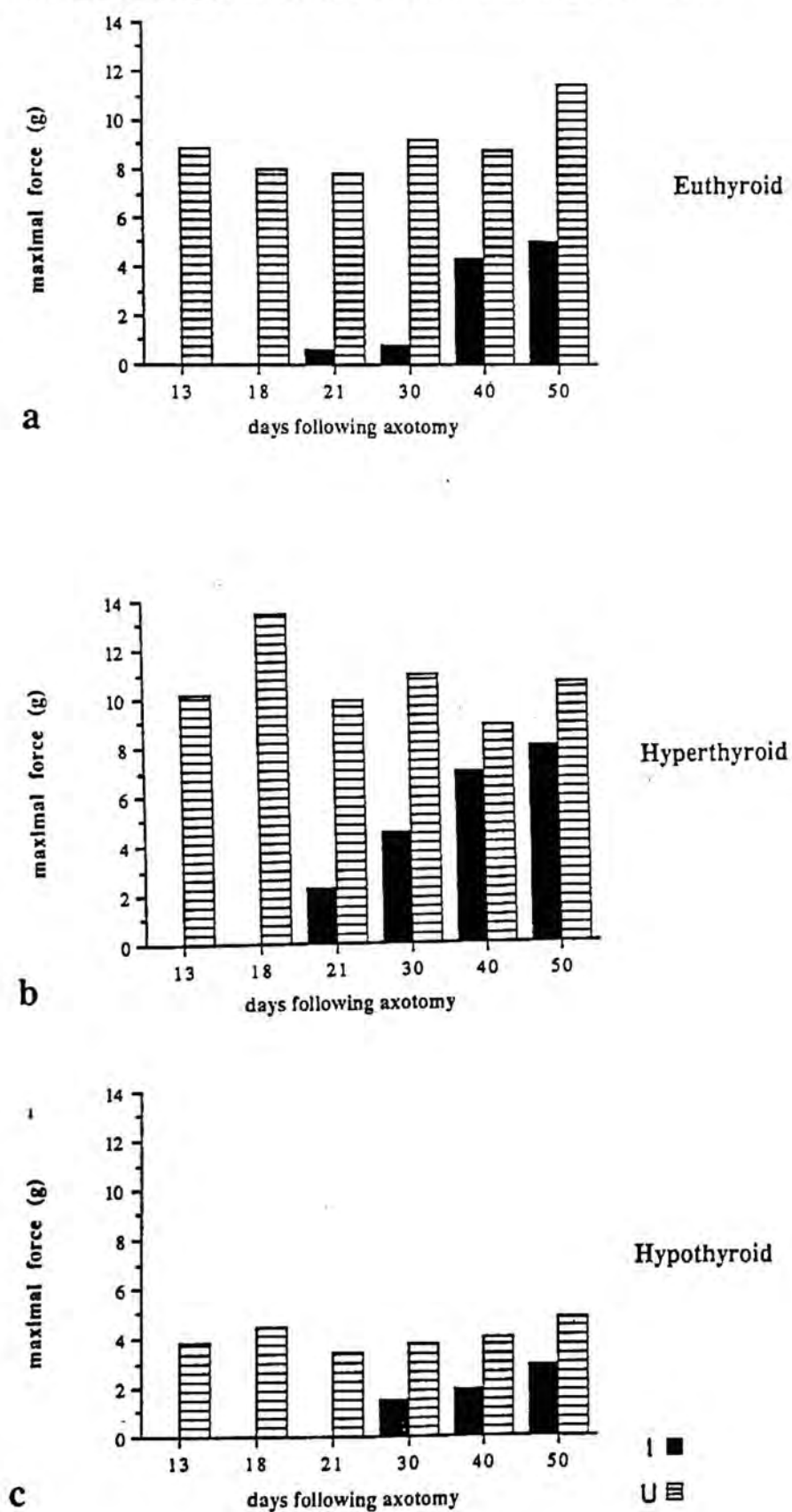
Maximal Tongue Force Produced following Axotomy

Figure 50

animals, stimulated tongue force was observed 30, 40 and 50 days following axotomy.

As depicted in Figure 51, increasing tongue force was evoked by incremental increases in stimuli voltages until a maximal value was obtained. The maximal force produced by stimulation of uninjured hypoglossal nerves averaged 12.4g among the hyperthyroid animals, similar to the euthyroid response (Fig 49).

A progressive increase in stimulated maximal force was observed 21 to 50 days following axotomy (Fig 50B). The recovery of maximal force production, expressed as the ratio of maximal force provoked following stimulation of the regenerated versus uninjured nerve, ranged from 0.25 at 21 days post-axotomy to 0.80, 50 days post-axotomy, among the hyperthyroid population.

Hypoglossal Nerve Function among the Hypothyroid Population

The recovery of stimulated force production by the tongue was not observed among the hypothyroid population until 30 days following axotomy (Table 5). Forty and fifty days following axotomy, all hypothyroid animals displayed tongue force production following nerve stimulation.

As depicted in Figure 52, small increases in tongue force were evoked by increases in stimuli voltages until a maximal value was obtained. The maximal force provoked by the stimulation of uninjured hypoglossal nerves averaged 5.0g among the hypothyroid animals, which is significantly

Figure 51. Twitch muscle force measured in the tongue over a series of increasing stimuli voltages among hyperthyroid animals. Each point represents an average from two animals, three measurements/animal.

Force Produced by Stimulation of Uninjured and Regenerated Hypoglossal Nerves in Hyperthyroid Animals

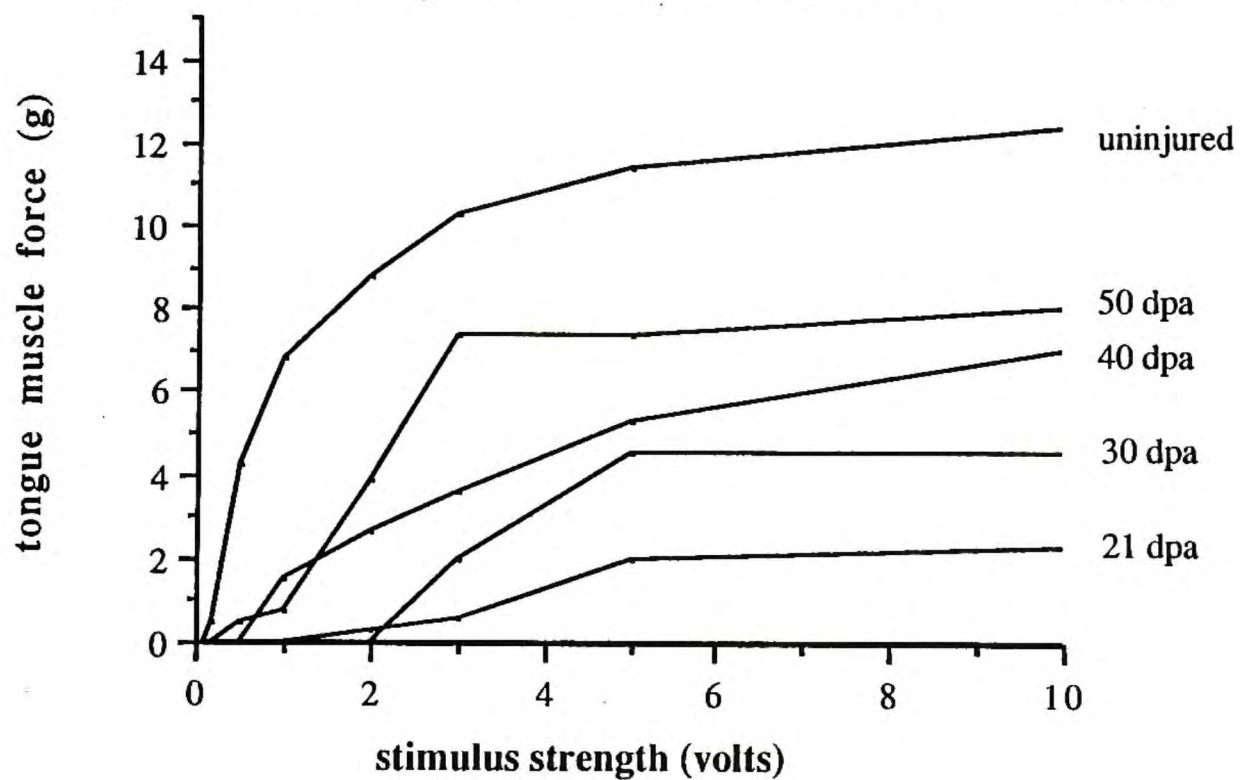


Figure 51

Figure 52. Twitch muscle force measured in the tongue over a series of increasing stimuli voltages among hypothyroid animals. Each point represents an average from two animals, three measurements/animal.

Force Produced by Stimulation of Uninjured and Regenerated Hypoglossal Nerves in Hypothyroid Animals

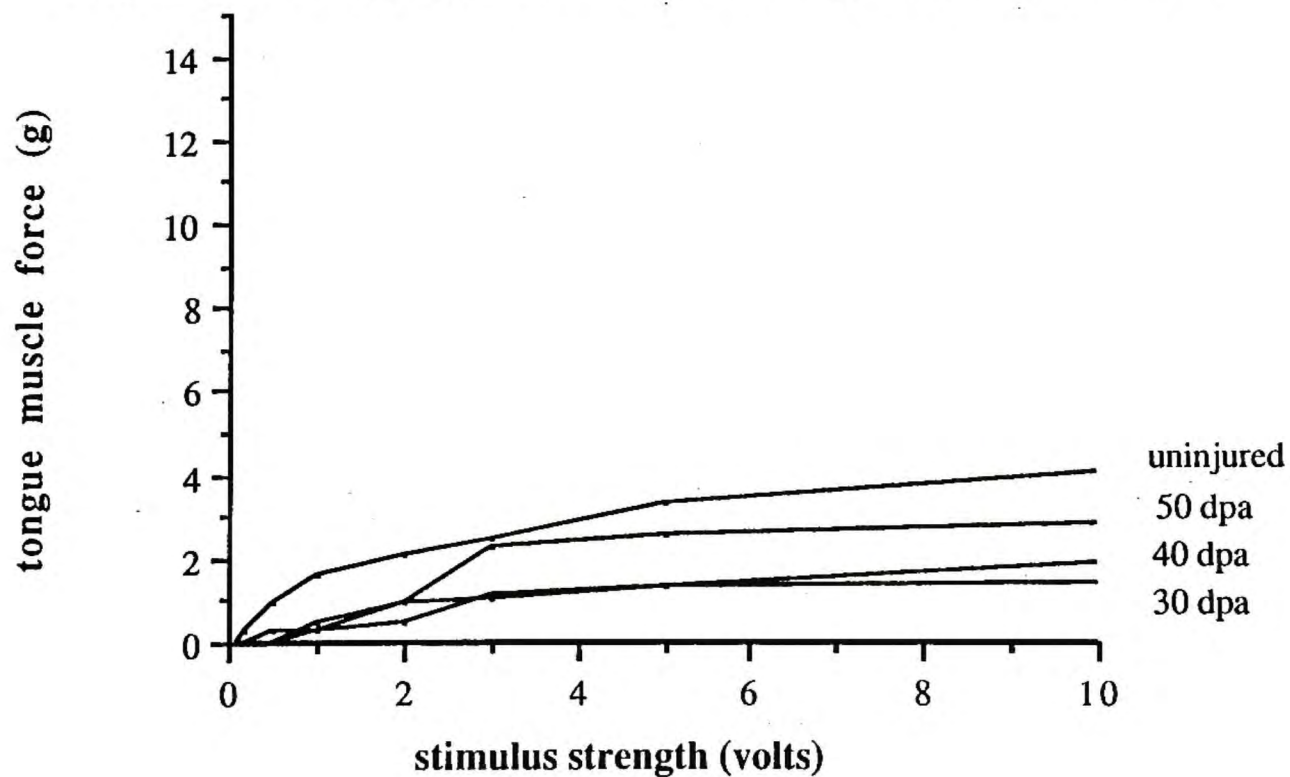


Figure 52

less than the eu- and hyperthyroid response ($p < 0.01$) (Fig 49).

Stimulated maximal force production by the tongue increased 21 to 50 days following axotomy among the hypothyroid animals (Fig 50C). The recovery of maximal force production, expressed as the ratio of maximal force provoked following stimulation of the regenerated versus uninjured nerve, ranged from 0.29 at 30 days post-axotomy to 0.55, 50 days post-axotomy.

DISCUSSION

The current work represents the first morphometric analysis of the somata and organelles from regenerating mammalian motoneurons. Following axotomy, hypoglossal nucleus volume was significantly diminished among the euthyroid animals. Concomitantly, all estimates of neuronal size indicated significant neuronal enlargement. Three intrasomatic organelles proved to be quantitatively altered by axotomy, including: the RER, perikaryal smooth membranes, and the nucleolus.

This study is the first examination of the influence of thyroid hormone status upon neuronal cell body structure following axotomy. Analysis of the structure of hypoglossal motoneurons from hyper- and hypothyroid animals revealed some unique retrograde responses among the two treatment groups. The influence of thyroid hormone status upon axonal regeneration also was examined. While hyperthyroidism failed to accelerate axonal regeneration in the current work, hypothyroidism was associated with delayed axonal elongation and functional reinnervation.

Use of Morphometry

The adaptation of morphometric techniques to the study of nervous tissue has evolved slowly during the last few decades and ultrastructural morphometric analyses are a recent development (Haug, 1982). The general principles of morphometry are historically rooted in the study of geology

and metallurgy (Weibel, 1973; Williams, 1977). Morphometric analysis engenders theoretical considerations based upon geometric probability. Also, there are special considerations imposed by the tissue of interest. In light of the paucity of morphometric data concerning neuronal ultrastructure, some discussion of the approach used in this study is appropriate.

Sound morphometric data rely upon superior and consistent preservation of tissue structure. In this study, tissue preparation (fixation, dehydration, embedding and sectioning) was handled according to well established techniques. Ultrastructural analysis of the tissue revealed good preservation of structure, as judged by the absence of mitochondrial swelling, uniform distribution of nuclear granular material and the continuity of cellular membranes (Hayat, 1970).

Obtaining a representative sample of the tissue is a pivotal aspect of morphometric analysis (Weibel, 1973). Careful consideration was given to each sampling level (animal, tissue section, cell, photomicrograph). Rats were age and sex matched in this study. Due to the highly oriented nature of the hypoglossal nucleus, a stratified sampling technique was employed, to minimize error (Weibel, 1973). Coronal sections of the hypoglossal nucleus were collected in order to maximize the amount of tissue available for volume measurement and electron microscopic evaluation. A systematic sample of these sections was

utilized for all analyses in order to eliminate rostral-caudal bias. Also, neurons were selected throughout the dorsal-ventral extent of each section in order to eliminate any bias in that plane.

Only neurons with a nucleus in the section were included in the sample. This nuclear bias, which was necessary for differentiating the cell body from the proximal dendrite (Peters et al., 1976), undoubtedly led to an overestimation of nuclear volume and surface area. Although correction methods for nuclear bias are available, they rely upon a constant nuclear position within the cell (William, 1977), a requirement not met by injured motoneurons. Sampling of the neuronal perikaryon was conducted in a systematic fashion as described previously (Loud, 1968).

A good sample not only lacks any spatial bias, but enjoys an adequate amount of data to assure its representative nature as well as statistical sensitivity (Weibel, 1973). The expensive and time-consuming nature of electron microscopic work, however, enforces some restriction on the sample size. Experiments with both maximal efficiency and sensitivity can be designed by analyzing the variance of preliminary data (Shay, 1975). Such an approach was utilized in this study. The number of animals, neurons and micrographs analyzed were sufficient, in this study, to reduce the percent standard error to less than 10% for all parameters except the smooth membrane

component, which exhibited a percent standard error of 14.7%. The sparse distribution of the smooth membranes within the perikaryon accounts for this higher percent standard error and is similar to estimates from a previous morphometric study (Buschmann & Manke, 1981). The number of statistical units used in this study conforms to those listed in the few morphometric analyses of neuronal structure following axotomy which have been reported (Barron, 1971; Dentinger et al., 1979; Barron, 1986).

The Morphometric Characterization of Regenerating Motoneurons

The following discussion focuses upon the quantified structural characteristics of regenerating motoneurons from euthyroid animals.

The Influence of Axotomy upon Hypoglossal Nucleus Volume

The present work is the first reported measure of hypoglossal nucleus volume. Previous studies have evaluated hypoglossal nucleus cross-sectional area following axotomy, however conflicting results were derived using that parameter (Sumner & Sutherland, 1973; Borke, 1983). In this study, the measurement of hypoglossal volume, a more sensitive parameter (Williams, 1977), revealed a significant reduction 7 to 30 days following axon transection.

Neuronal cell bodies, glia and neurites are located within the hypoglossal nucleus. A decrease in any of these

components could theoretically lead to a reduction in hypoglossal nucleus volume. In this study, neuronal cell body size increased following axotomy, as previously observed (Aldskogius et al., 1980). Also, the number of neuronal cell bodies was constant in this study. In addition, glial cell proliferation within the hypoglossal followed axotomy (Borke, 1982; Reisert et al., 1984). Therefore, a decrease in total neuronal cell body or glia volume cannot account for the diminished hypoglossal nucleus volume.

The reduction in hypoglossal nucleus volume observed in this study probably reflects dendritic retraction by the axotomized motoneurons. There is direct evidence for dendritic retraction. In an earlier study, Golgi-stained hypoglossal motoneurons of the rat demonstrated a reversible decrease in dendritic fields 10 to 50 days following hypoglossal nerve transection (Sumner and Watson, 1971). At the ultrastructural level, diminished dendritic cross-sectional area was observed within axotomized hypoglossal nuclei (Sumner & Sutherland, 1973). The stimulus and advantage of dendritic retraction remain obscure. Partial deafferentation of the injured motoneuron is temporally coincident with the reduction in dendritic field (Sumner & Sutherland, 1973; Borke, 1982), and the disrupted functional connection between the neuron and its efferent target (Sumner and Watson, 1971). Thus, afferent and efferent connections may modulate dendritic size.

The fluctuating hypoglossal nucleus volume deserves special methodological consideration. In this study, ratio and absolute estimators were utilized. The use of ratios alone, such as neuronal fractional volume (percent of hypoglossal nucleus volume composed of neuronal cell bodies), when the reference volume is unknown, can lead to dubious conclusions (Braendgaard & Gundersen, 1986). For example, decreased hypoglossal nucleus volume and constant neuronal volume would lead to an elevation of the neuronal fractional volume estimate. To conclude that neuronal volume did increase, in this case, is erroneous. Therefore absolute estimators, such as total neuronal volume, were derived in this study.

Axotomy Induced Neuronal Enlargement

Neuronal swelling, evoked by axotomy, is a classical component of the retrograde reaction and an imputed sign of regenerative activity (Lieberman, 1971). Two to three weeks following axotomy, all parameters estimating neuronal size demonstrated a maximal increase of 10 to 25% in this study, confirming a previous report (Aldskogius et al., 1980). This pattern of neuronal enlargement is discordant with an earlier survey, which reported as much as a 50% increase in neuronal volume following axotomy (Watson, 1968). However, the discrepancies could reflect different tissue fixation and preparation methods. In the latter study the inferior method of immersion fixation was utilized (Hayat,

1981).

In the current study, uninjured neurons constituted 12.8% of the hypoglossal nuclear volume, slightly larger than an earlier estimate of 8.4% (Sumner & Sutherland 1973). In the previously published study, the sampling method was not directed toward estimation of neuronal volume, and by the authors' own admission, probably underestimated that parameter. In another previous study, in which the neuronal fractional volume of the hypoglossal nucleus was calculated from random ultrathin sections, an estimate of 11.7% was reported (Reisert et al., 1984). The estimates of neuronal fractional volume from the present study were subjected to the Holmes' effect which leads to overestimation, and opposing tissue contrast effects which cannot be corrected (Williams, 1977). Despite these effects, an estimate was achieved in this study that is nearly identical to previously published measurements. In addition, the methods used in this study, which allowed measurement of the entire hypoglossal nucleus cross-sectional area, were sensitive to changes in neuronal fractional volume following axotomy. The previous measurements of neuronal fractional volume in the axotomized hypoglossal nucleus did not detect neuronal enlargement due to the unsubstantiated assumption that hypoglossal nucleus volume was constant (Reisert et al., 1984). The elevated neuronal fractional volume produced by axotomy, and observed at each time point analyzed following axotomy, reflects both increased cell volume and diminished

hypoglossal nucleus volume.

The Response of the Cell Nucleus to Axotomy

In the current study, 10% and 35% of the motoneurons examined 7 and 13 days following axotomy, respectively, contained eccentric nuclei with a ruffled envelope, confirming and extending previous reports (Kirkpatrick, 1968; Lieberman, 1971; Fernando, 1973; Borke, 1983).

Although invagination of the nuclear envelope was evident among some motoneurons 7 and 13 days following axotomy, the total nuclear volume and surface area were not significantly altered following axotomy. An earlier study also reported that nuclear cross-sectional area was constant among axotomized hypoglossal motoneurons (Aldskogius et al., 1980). While the surface area of the nuclear envelope may indeed increase within a particular neuron, estimates from the entire neuronal population do not indicate a general or substantial overall increase in nuclear surface area. Apparently, the regulation of nuclear volume and surface area following axotomy is variable among different neuronal populations, as well as among individual neurons within a single population. Quantitative data from other neuronal populations have demonstrated constant (Whitnall and Grafstein, 1982, 1983), diminishing (Barron et al., 1982) and augmented (LaVelle & LaVelle, 1958; Jones & LaVelle, 1985) nuclear size following axotomy. Among facial motor neurons of the hamster, the perimeter of the cell nuclei

proved constant following axotomy (Jones & LaVelle. 1985).

The Nucleoli of Regenerating Neurons

The dramatic increase in nucleolar volume exhibited by hypoglossal motoneurons 7 days following axotomy confirms previous quantitative analyses (Watson, 1965; Aldskogius et al., 1980). Additionally, nucleolar vacuolization and eccentricity observed qualitatively in this study concur with prior reports (Lieberman, 1971; Rees, 1971). These nucleolar responses, which are characteristic of regenerating neurons (Lieberman, 1971), represent one of the few structural changes experimentally correlated to metabolic activity. Specifically, increased rRNA synthesis, transport of RNA from the nucleus to the cytoplasm and subsequent protein synthesis have been demonstrated among regenerating hypoglossal motoneurons (Watson 1965, 1968), as well as facial motor neurons of the hamster (LaVelle & LaVelle, 1958) and RGC of the goldfish (Murray & Grafstein, 1969; Whitnall & Grafstein 1982, 1983).

The Endoplasmic Reticulum of Chromatolytic Neurons

Axotomy induced RER disruption has emerged as the hallmark of the axon reaction (Lieberman, 1971). Although altered cytoplasmic basophilia, or chromatolysis, was initially reported in 1892 (Nissl, 1892) and has been extensively studied since then, "little important information on chromatolytic phenomena have been obtained

due to ... a simple qualitative approach (Lieberman, 1971)." Clearly, disruption of the RER occurs following axotomy (Kirkpatrick, 1968; Lieberman, 1971; Fernando, 1973; Borke, 1983); alterations in the amount of RER following axotomy and its functional significance are unknown.

This study has provided the first quantitative analysis of RER surface area within regenerating mammalian neurons. RER surface density diminished 30% seven days following axotomy among hypoglossal motoneurons, temporally coincident with the maximal qualitative disruption of the RER (Kirkpatrick, 1968; Borke, 1983). By 21 days following the axonal lesion, RER surface density among axotomized neurons was similar to that measured among uninjured neurons. Qualitative reorganization of the RER was apparent 21 days following the lesion.

A quantitative examination of the RER has been reported for one other population of regenerating neurons, the RGC of the goldfish (Whitnall & Grafstein, 1982, 1983). In that study, the fractional volume of the RER was derived. Although fractional volume estimates of a membranous organelle are less suitable than surface area measurements (Buschmann & Manke, 1981), and direct comparison among the data is precluded by the use of different approaches, the trends among the two regenerating systems can be compared. The axotomized RGC exhibit a temporary, modest depression of the RER fractional volume which mirrors the RER surface area fluctuations within regenerating hypoglossal motoneurons

(Whitnall & Grafstein, 1982, 1983). Specifically, the RER fractional volume of the RGC was diminished by approximately 25% six days following optic tract lesion, returning to control levels 13 days post-axotomy.

The RER also has been quantitatively evaluated among two non-regenerating neuronal populations, the rat RGC (Barron et al., 1986) and the feline Betz cell (Dentinger et al., 1979). These two populations of neurons exhibit a dramatic disruption of the qualitative appearance of the RER but only a modest reduction in the amount of RER similar to the response of the hypoglossal motoneuron. All of these data suggest that axotomy fails to elicit an extreme or chronic depletion of the RER despite qualitative disruption.

Coincident with the temporary depletion of ribosome-studded endoplasmic reticulum, is the proliferation of smooth endoplasmic reticulum. The smooth membrane quantified in this study was composed of vesicular and tubular cisterns and constituted less than 5% of the total perikaryal membranous organelles within uninjured neurons. Subsurface cisterns were not included due to insufficient sampling. In present work, the smooth membrane surface area of hypoglossal motoneurons increased more than 200% seven days following axotomy. Smooth membrane surface area was similar to that observed in the uninjured population 21 to 30 days following axotomy. Many previous studies have failed to report this disturbance, probably due to the difficulty of qualitatively assessing a sparsely distributed

organelle. It is interesting that the increase in smooth membrane surface area temporally corresponded to the RER depletion and accounted for 50% of the change, suggesting that ribosome denuding of the RER, whether due to disruptive or anabolic events, may occur (Whitnall & Grafstein, 1982, 1983).

The regenerating retinal ganglion cell from the goldfish is the only other system in which the smooth endoplasmic reticulum has been quantified (Whitnall & Grafstein, 1982, 1983); the fractional volume was estimated, as described for the RER. A similar pattern of smooth membrane hyperplasia, as observed among regenerating hypoglossal motoneurons, was detected among the retinal ganglion cells.

The disorganization, denuding and/or depletion of the RER among axotomized neurons have been attributed to non-specific disruptive events that follow injury (Grafstein, 1983). One trigger for chromatolysis which has been proposed is water influx and subsequent neuronal swelling (Cragg, 1970; Grafstein, 1983). The structural data from this and other studies do not support this hypothesis. For example, neuronal enlargement does not appear to precede RER depletion among hypoglossal motoneurons, as measured in this and a previous study (Aldskogius et al., 1980). Also, among some chromatolytic populations, neuronal size diminishes following axotomy (Dentinger et al., 1979; Barron et al., 1986).

Additionally, neuronal swelling should produce a dilution of all organelles, including the RER, within the perikaryon; an effect not evidenced by this or other studies (Whitnall & Grafstein, 1982, 1983). Thus, water influx does not appear to be associated with chromatolysis.

The quantitative data from this study provide new information about the structural response of the endoplasmic reticulum to axotomy. The amount and configuration of the endoplasmic reticulum have long been heralded as an important indicator of cell function, but the metabolic significance of the chromatolytic changes is not fully understood. Functions which have been assigned to the endoplasmic reticulum include protein synthesis (Palade, 1975), biomembrane formation (Holtzman & Mercurio, 1980), lysosome generation (Novikoff et al., 1971), and, in some tissues, calcium sequestration (MacLennan et al., 1983). Modification of any of these processes could feasibly influence the appearance of the endoplasmic reticulum within a neuron.

The protein synthetic function is the best defined of the RER properties (Palade, 1975). The disrupted appearance of the RER among axotomized neurons has therefore been viewed as the structural correlate of transformed protein synthetic activity (Lieberman, 1971; Grafstein, 1983). Axotomy does induce significant changes in the proteins synthesized by the neuron, presumably by altering mRNA species transcribed in the cell. Proteins which

function in synaptic transmission and are synthesized on RER (Flumerfelt & Lewis, 1975; Rotter et al., 1977; Wooten et al., 1978; Rotter et al., 1984) are substantially diminished in axotomized neurons. Among RGC of the goldfish, diminished labeling of the RER with newly synthesized proteins occurred 6 days following axotomy, during the process of axonal elongation; thirty days following axotomy, during synaptogenesis, RER labeling was accentuated (Whitnall & Grafstein, 1982, 1983). The data from the current study also indicated that the total surface area of the RER was reestablished to control levels prior to functional recovery.

The rough and smooth endoplasmic reticulum, within the neuronal cell body, is also the site of biomembrane synthesis (Holtzman & Mercurio, 1980). This synthetic role of the endoplasmic reticulum in axotomized neurons, however, remains unexplored. Certainly the formation of new plasma membrane is essential for axonal elongation. It is interesting that current theories suggest the smooth endoplasmic reticulum may serve as a membrane shuttle between the RER, Golgi apparatus and plasma membrane (Holtzman & Mercurio, 1980). The RER depletion and concomitant smooth membrane proliferation, detected in this study and within the RGC of the goldfish during axonal elongation, may be the manifestation of prolific membrane formation (Whitnall & Grafstein, 1982, 1983).

Lysosome formation has been attributed to regions of

the endoplasmic reticulum which share enzymatic properties with lysosomes (Novikoff et al., 1971). In fact, the smooth membrane proliferation within axotomized neurons has been ascribed to escalated lysosome production (Lieberman, 1971). Although the lysosomal volume of neurons was constant following axotomy in this and other studies (Dentinger et al., 1979; Whitnall & Grafstein, 1982, 1983), an increase in lysosome formation by smooth membrane cannot be ruled out. Hyperplastic smooth membrane detected in this study could, therefore, indicate an increase in lysosome synthesis.

The endoplasmic reticulum is celebrated for its role of calcium sequestration within muscle (MacLennan et al., 1983), and evidence for a similar function within neuronal systems has been advanced (Henkart et al., 1978; Andrews et al., 1988). An increase of intraneuronal calcium has been correlated with endoplasmic reticular swelling and the appearance of flocculent matter within individual cisterns, not unlike that observed during chromatolysis (Henkart et al., 1978). Intraneuronal calcium storage within the endoplasmic reticulum has not been experimentally analyzed following axotomy.

The potential functions of the endoplasmic reticulum within neurons are largely unexplored (Holtzman & Mercurio, 1980). The relationship of these activities with the structural configuration of the endoplasmic reticulum is obscure, thus masking the functional significance of chromatolysis.

Response of the Golgi Apparatus to Axotomy

The Golgi apparatus response to axotomy has long been controversial (Lieberman, 1971). While retispersion (peripheral displacement) and retisolution (fragmentation) of the Golgi apparatus among axotomized neurons were initially demonstrated using histochemical techniques, electron microscopic studies generally have failed to verify such responses, except for studies of spinal motor neurons of the cat (Barron et al., 1971; Lieberman, 1971). The discrepancies among most ultrastructural and histochemical data were attributed to the vagaries of histochemistry (Lieberman, 1971). In an ultrastructural analysis of hypoglossal motoneurons from the rat the "prominence of the Golgi zones" following axotomy was described (Kirkpatrick, 1968). Qualitative observations of the Golgi apparatus in this and a previous report (Borke, 1983) have failed to corroborate the earlier qualitative observation. No significant alteration in the Golgi apparatus surface density or area was detected following axotomy in the present study. Additionally, among the regenerating RGC of the goldfish (Whitnall & Grafstein, 1982, 1983) and non-regenerating Betz cells of the cat (Dentinger et al., 1979), the volume fraction of the Golgi apparatus was unchanged following axotomy. Therefore, all quantitative analyses so far have not supported the occurrence of axotomy-induced surface density/fractional volume changes of the Golgi apparatus.

Although this study and others have failed to demonstrate quantitative alterations of the Golgi apparatus following axotomy, increased routing of newly synthesized proteins to the Golgi apparatus was demonstrated in regenerating RGC of the goldfish 6, 14 and 30 days following axotomy (Whitnall & Grafstein, 1982, 1983). Thus, in the face of a constant Golgi apparatus fractional volume, enhanced metabolic activity was apparent among regenerating RGC.

Lysosomal Response of Regenerating Neurons

Lysosomes are polymorphic organelles which contain many acid hydrolases (de Duve, 1983). Previous qualitative analyses have described lysosomal (or dense bodies) proliferation as well as hypertrophy of individual lysosomes (Lieberman, 1971) among axotomized neurons, including hypoglossal motoneurons (Kirkpatrick, 1968; Borke, 1983). Also, many histochemical studies have detected an increase in acid phosphatase within axotomized neurons (Lieberman, 1971).

Although an occasional neuron appeared to contain many lysosomes, lysosomal volume was not significantly altered following axotomy. Unchanging lysosomal volume following axotomy has also been quantitatively demonstrated in three other systems including spinal motor neurons of the cat, RGC of the goldfish and Betz cells of the cat (Barron et al., 1971; Dentinger et al., 1979; Whitnall & Grafstein,

1982, 1983). In all neuronal systems, lysosomal fractional volume constituted only 1% of the perikaryon.

How can the discrepancy between qualitative observations and quantitative data concerning lysosomes be reconciled? In the present investigation, a few axotomized neurons rich in lysosomes were observed. Lysosomal proliferation may be restricted to a minority of the neuronal population, and thus evade quantitative detection.

Many of the early reports concerning lysosomal proliferation following axotomy relied upon acid phosphatase activity as a marker (Lieberman, 1971). Acid phosphatase activity may denote the presence of lysosomes as well as smooth endoplasmic reticulum and some Golgi complexes (Novikoff et al., 1971). Therefore, an increase in acid phosphatase activity in the absence of lysosomal proliferation is possible. Direct evidence for this idea is derived from an evaluation of degenerating neuroblasts, in which a decrease in lysosomal number was coincident with an increase in acid phosphatase activity (Pannesse et al., 1976).

Smooth endoplasmic reticulum containing acid phosphatase activity is considered a precursor of primary lysosomes (Novikoff et al., 1971). Proliferation of the smooth membrane with acid phosphatase activity suggests subsequent lysosomal proliferation, which is not observed in the perikaryon. However, an increase in reactive dendrites packed with lysosomes, within the axotomized hypoglossal

nucleus has been reported (Borke, 1979). Such profiles suggest remodeling of the dendrites. This activity may account for the diminished dendrite and hypoglossal nucleus size as well as any increase in lysosomal formation within neurons.

The Response of Neuronal Mitochondria to Axotomy

While some reports suggest that axotomy induces mitochondrial proliferation, swelling and hypertrophy, other qualitative and quantitative ultrastructural studies and have not confirmed these observations (Lieberman, 1971). In a previous examination of hypoglossal motoneurons 6 days following axotomy, mitochondrial fractional volume did not differ from the uninjured control estimates (Kirkpatrick, 1968). The data from the present study extend that observation; mitochondrial volume was not significantly altered 7 to 30 days following axotomy. Also, in this study it was estimated that mitochondria composed approximately 8% of the perikaryal volume, similar to the previous estimate of 8.3% (Kirkpatrick, 1968). Mitochondrial fractional volume was also found to be constant among axotomized Betz cells of the cat (Dentinger et al., 1979). Among RGC of the goldfish, mitochondrial fractional volume significantly diminished following axotomy; however, the tremendous perikaryal enlargement may account for this alteration (Whitnall & Grafstein, 1982, 1983).

Comparison of Morphometric Data from Regenerating Neurons

Many of the retrograde responses of hypoglossal motoneurons quantified in this study and retinal ganglion cells from the goldfish are similar (Whitnall & Grafstein, 1982; Whitnall & Grafstein, 1983). Neuronal and nucleolar enlargement, smooth membrane proliferation and a modest depletion of the RER characterize the cell body reaction in both populations. The striking resemblance of the retrograde reaction within these disparate neuronal populations, elucidated by morphometric techniques, suggests that cell body responses to axotomy may be more highly conserved than previous qualitative data has indicated.

Regenerating spinal motor neurons of the cat have been examined quantitatively to a lesser extent than either of the above mentioned neuronal systems. The available data have indicated that neuronal and nucleolar enlargement are absent among spinal motoneurons of the cat and the kitten (Cova & Barron, 1981; Barron et al., 1982). These results suggests that several cellular strategies for peripheral nerve regeneration exist among different mammalian species. The disparity among these neuronal systems may also reflect, in part, different time courses of regeneration and reinnervation. In the present work, hypoglossal motoneurons of the rat demonstrated functional recovery 21 days post-axotomy; retinal ganglion cells of the goldfish also achieve functional recovery 3 to 4 weeks following axotomy (Grafstein & Murray, 1969). In contrast, spinal motoneurons

of the cat do not reach their target until 60 to 90 days following axonal transection (Cova & Barron, 1981; Barron et al., 1982). This extended period of axonal elongation and separation from the synaptic target experienced by spinal motoneurons of the cat may influence the cell body response (Lieberman, 1974; Johnson et al., 1985).

The morphometric techniques used in the current study have afforded precise identification of organelle responses and facilitated the comparison of data among the distinct populations. Extended morphometric analyses of regenerating mammalian neuronal systems, may further reveal which cellular events are non-specific reactions to injury, contributors to regeneration, or indicators of reinnervation.

The Influence of Thyroid Hormone Status upon the Retrograde Response of Regenerating Hypoglossal Motoneurons

The influence of thyroid hormone status upon the structure of regenerating hypoglossal motoneurons was examined. Several studies have evaluated the effect of hyper- and hypothyroidism upon axonal regeneration. The ultrastructure of axotomized neurons from hyper- and hypothyroid animals has not been reported previously.

The Influence of Altered Thyroid Hormone Status and Axotomy upon Hypoglossal Nucleus Volume

The average volume of uninjured hypoglossal nuclei

from hyper- and euthyroid animals was similar. Following axotomy, a significant reduction in hypoglossal volume, detected 7 to 30 days post-axotomy among the euthyroid population, was not apparent among the hyperthyroid population until 30 days following the axon lesion. The cause of the delayed hypoglossal nucleus volume reduction, induced by hyperthyroidism, is unknown. Qualitative analysis of the neuropil, at the light microscopic level, failed to reveal any differences within the hypoglossal nuclei from hyper- and euthyroid rats. A quantitative examination of the neuropil may expose particular components which respond differently to axotomy in the presence of excessive circulating thyroxine.

The average volume of uninjured hypoglossal nuclei from hypothyroid animals was significantly less than measured among hyper- and euthyroid groups. The diminished hypoglossal volume among the hypothyroid animals does not reflect a reduction in neuronal cell body size, since the average neuronal cross-sectional area and maximal diameter were similar among all uninjured hypoglossal nuclei, regardless of thyroid hormone status. Also, the total neuronal volume within uninjured hypoglossal nuclei was similar among the eu-, hyper- and hypothyroid animals, suggesting that neuronal cell body number was comparable among the three groups. Examination of hypoglossal nuclei at the light microscopic level did not expose any qualitative differences in the neuropil from hypothyroid

animals when compared with the other two groups. Hypothyroidism can retard dendritic formation and myelination during development (Nunez, 1984). A previous work has shown that an adult-like neuropil is present within the hypoglossal nucleus of the rat, 24 days post-natal, and the cross-sectional area of the hypoglossal nucleus largely achieves its adult size by 20 days post-natal (Borke, 1979). The hypothyroidism induced 21 days postnatal in this study, may have impaired the terminal phase of hypoglossal nucleus maturation. Quantitative analysis of the neuropil composition may elucidate the source of the diminished hypoglossal volume among hypothyroid animals.

Among the hypothyroid animals, the post-axotomy reduction in hypoglossal nucleus volume was similar to the response evidenced by euthyroid animals. The estimated volume of hypoglossal nuclei from hypothyroid animals significantly decreased 10% to 15% from 7 to 30 days following axotomy. Thus, despite an initial volume deficit, the hypoglossal nuclei from hypothyroid animals were also capable of further contraction following axotomy.

Neuronal Size Following Altered Thyroid Hormone Status and Axotomy

Estimates of the volume, cross-sectional area and maximal diameter of uninjured neurons from hyper- and euthyroid animals were similar. Neuronal cell body enlargement, characteristic of the euthyroid population,

also was observed among hyperthyroid animals 7 to 21 days following axotomy. Although neuronal enlargement was no longer evident 30 days following axotomy among the euthyroid animals, all parameters estimating neuronal size indicated maximal neuronal hypertrophy 30 days following axotomy among the hyperthyroid population. The accentuated neuronal hypertrophy, induced by axotomy, may reflect enhanced neuronal metabolism among the hyperthyroid population. The thyroxine treatment augmented neuronal cell body enlargement subsequent to functional reinnervation (21 dpa), suggesting that the interaction of the neuron with the reinnervated muscle may have been influenced by the hyperthyroid status.

Among the hypothyroid animals, the volume, cross-sectional area and maximal diameter of uninjured neurons were similar to that observed among corresponding eu- and hyperthyroid populations. Axotomy failed to induce neuronal cell body enlargement among the hypothyroid population. Since the neuronal hypertrophy which follows axotomy is thought to be an anabolic response (Lieberman, 1971), its absence suggests that hypothyroidism compromises the regenerative capacity of hypoglossal motoneurons.

The Influence of Thyroid Hormone Status and Axotomy upon the Cell Nucleus

Among the hyperthyroid animals, axotomy provoked nuclear eccentricity and nuclear envelope ruffling. The maximal nuclear response to axotomy was observed 7 days

following the axon lesion, when 45 to 55% of the neurons examined contained eccentrically displaced nuclei with undulating surfaces. Thirteen days post-axotomy, 30 to 40% of the neurons demonstrated the nuclear alterations. Although the percent of neurons containing eccentric and ruffled nuclei following axotomy was enhanced among the hyperthyroid animals versus the euthyroid population, estimates of cell nuclear volume and surface area were not significantly different among the uninjured and axotomized hyperthyroid groups. The estimated volume and surface area of nuclei from uninjured and axotomized neurons from the hyperthyroid population were similar to corresponding values from euthyroid animals.

Nuclear eccentricity and invagination of the nuclear envelope were dramatically expressed by axotomized motoneurons from the hypothyroid population. These nuclear alterations were observed among 95% of the motoneurons examined 7 days following axotomy. The enhanced nuclear eccentricity and ruffling evidenced by injured neurons from the hypothyroid group corresponded to a significant increase of nuclear surface area 7 days post-axotomy. Nuclear volume and surface area among uninjured neurons from the hypothyroid group were similar to the corresponding eu- and hyperthyroid values, as were all post-axotomy nuclear volume measurements and nuclear surface area measurements 13 to 30 days post-axotomy.

Axotomized motoneurons from hyper- and hypothyroid

animals occasionally contained nuclear filaments. Such structures were never observed among the uninjured neurons from the hyper- and hypothyroid populations, nor in any motoneuron from the euthyroid group. The presence of nuclear filaments within uninjured or axotomized hypoglossal motoneurons of the rat has not been previously reported. Nuclear filaments have been observed among many cell types (Feldman & Peters; 1972). Electrical stimulation (Seite et al., 1973), cocaine administration (Johnson & Weissman, 1988), and increasing age (Feldman & Peters; 1972) reportedly increase the number of nuclear filaments within various neuronal populations. Current theories suggest that nuclear filaments form in response to "a high level of demand on the cell's metabolic machinery" (Feldman & Peters, 1972). Regenerating motoneurons from hyper- and hypothyroid animals may experience greater metabolic stress than corresponding cells from the euthyroid population. A more extensive survey of hypoglossal motoneurons is necessary to determine the true frequency of nuclear filaments among uninjured and axotomized motoneurons from eu-, hyper- and hypothyroid animals.

Nucleolar Size and Structure Following Altered Thyroid Hormone Status and Axotomy

The nucleolar fractional and total volume from uninjured motoneurons of hyper- and hypothyroid animals were not significantly different than corresponding measurements

from the euthyroid population. However, following axotomy, nucleolar enlargement, observed in the euthyroid population, was absent among both the hyper- and hypothyroid animals. The failure to detect this classic retrograde response to axotomy among the hyper- and hypothyroid populations was surprising. Nucleolar enlargement is considered the ultrastructural correlate of the enhanced RNA and protein synthesis underlying axonal regeneration (Lieberman, 1971). Could the data indicate that enhanced protein synthesis is absent among axotomized motoneurons from the hyper- and hypothyroid animals?

The present experiment cannot directly answer this question. Qualitative ultrastructural data do suggest that altered nucleolar metabolism following axotomy occurs among the hyperthyroid group. Large nucleolar vacuoles were observed among the axotomized motoneurons from hyperthyroid animals. Nucleolar vacuoles appear to be involved in the storage and transport of ribosomal precursors synthesized within the nucleolus (Moreno Diaz de la Espina et al., 1980; Goessens, 1984). Heightened nucleolar metabolism and paradoxically, impairment of nucleolar activity have been shown to induce vacuole formation (Moreno Diaz de la Espina et al., 1980). The appearance of the large nucleolar vacuoles among regenerating motoneurons from hyperthyroid animals suggests that, in spite of no overall enlargement of the nucleolus, altered nucleolar function may have resulted from the axotomy of the thyroxine treatment group.

Among the hyperthyroid population, multiple nucleoli were sometimes present within injured neurons. Multiple nucleoli were not observed among the hypo- or euthyroid neuronal populations. The increase in nucleolar number may hint that the axotomized motoneurons from the hyperthyroid animals are metabolically altered (Goessens, 1984). Only a single previous report has described the rare occurrence of multiple nuclei within hypoglossal motoneurons (Watson, 1968). A larger neuronal sample to investigate true nucleolar frequency would be necessary for validation of this result.

Although nucleolar enlargement did not occur following axotomy among the hypothyroid animals, the organization of nucleolar structure was similar among the eu- and hypothyroid populations following axon transection. Specifically, small nucleolar vacuoles were often observed 7 and 13 days following axotomy, within the hypothyroid population. This observation, as well as the qualitative impression of increased free polyribosomes within the cytoplasm of axotomized neurons from hypothyroid animals, implies that axotomy induced an alteration in protein synthesis among this population. The axotomized neurons from the hypothyroid population may have staged a restricted response to axotomy, as the absence of nucleolar enlargement suggests. Decelerated axonal elongation among this population provides additional evidence for a compromised regenerative response. Molecular and biochemical evaluation

of axotomized motoneurons from hypothyroid animals are needed to resolve this issue.

THE INFLUENCE OF THYROID HORMONE STATUS UPON AXONAL REGENERATION

The progress of axonal regeneration among eu-, hyper-, and hypothyroid animals was analyzed by assessing retrograde labeling of neuronal cell bodies with HRP and nerve stimulated muscle force production.

Retrograde Labeling of Motoneurons with Horseradish Peroxidase

The retrograde transport of HRP from the innervation target to neuronal cell body is a cellular process which is commonly exploited to trace neuronal connectivity (LaVail, 1975; Mesulam, 1982). HRP labeling of the neuronal cell body, in the present study, relied upon full extension of the regrowing axons to the tongue.

Sixty-four percent of the uninjured motoneurons examined, from euthyroid animals, contained HRP. (Among two euthyroid and one hyperthyroid animals, less than 30% of the uninjured hypoglossal motoneurons contained reaction product. These animals were not included in the analysis, since the HRP injection or tissue processing may have produced the diminished labeling.) Similar to the euthyroid response, 67% of the motoneurons from the uninjured hypoglossal nuclei of hypothyroid animals were HRP-positive.

Among the hyperthyroid animals, the percentage of uninjured motoneurons retrogradely labeled with HRP was significantly less than observed in the eu- (64%) or hypothyroid (67%) animals, averaging 51% ($p < 0.05$). Since the injection protocol and tissue processing were handled in a consistent fashion, the diminished HRP labeling may reflect a physiological effect of thyroxine treatment upon HRP metabolism. Many factors can impinge upon the retrograde transport and reaction of HRP within neurons, including the amount of HRP available to the nerve terminals, innervation density of the injection site, terminal activity, pathway length, and neuronal transport and degradation of HRP (LaVail, 1975; Mesulum, 1982). The influence of excessive thyroxine upon the effectors of HRP metabolism is not known.

Among the euthyroid population, retrograde HRP labeling of motoneurons was first detected 18 days following axotomy, heralding the invasion of the tongue by regenerating axons. This is comparable to a previous report, in which hypoglossal motoneurons with tongue-injected HRP were observed by 3 weeks post-axotomy (Yu & Srinivasan, 1981).

Among the euthyroid animals, 40% of the neurons examined were HRP-positive 18 days following axotomy; 50 days post-axotomy, 54% of the hypoglossal neurons examined contained the peroxidase reaction product. The percentage of HRP positive neurons in the injured hypoglossal nucleus

ranged from 25 to 75% of those within the uninjured contralateral nucleus 18 to 50 days post-axotomy. These data which indicate diminished labeling of the injured versus contralateral uninjured nucleus could result from altered uptake or processing of HRP by regrowing axons, asynchronous axonal regeneration or the failure of some neurons to reinnervate the tongue. Neurons which do not reinnervate the tongue may ultimately degenerate. Although axotomy-induced neuronal loss was not observed among any of the thyroid hormone treatment groups in this study, neuronal death at later post-axotomy intervals, as previously reported (Aldskogius et al., 1980), may occur.

HRP was first identified in axotomized neuronal cell bodies 18 days following nerve transection among the hyperthyroid animals, matching the euthyroid response. These data confirm a previous study (Yu & Srinivasan, 1981), in which hyperthyroidism failed to detectably accelerate hypoglossal nerve elongation. Although it has been reported that thyroid hormone treatment accelerates axonal elongation, these studies relied upon histological assessment of the advancing myelinated fibers (Cockett and Kiernan, 1973; Danielsen et al., 1986). Thyroxine-stimulated myelination, which has been described (Stelmack & Kiernan, 1977), may have led to the impression of accelerated regeneration. More sensitive measures of sciatic nerve elongation have failed to substantiate the claim of accelerated axonal elongation following thyroid

hormone treatment (Berenberg et al., 1977; Cotrufo et al., 1979; Allpress & Pollock, 1986).

Among hyperthyroid animals, 30% of the injured neurons examined were labeled with HRP, 18 days following axotomy and any further augmentation of the percent neuronal labeling was not observed through 50 days post-axotomy. Thus, as recorded among the uninjured populations, there was diminished HRP labeling of injured neurons in the hyperthyroid versus euthyroid groups. The ratio of HRP positive neurons in the injured versus uninjured hypoglossal nucleus averaged 51%, 18 to 50 days following the nerve lesion. A previous report indicated that hyperthyroidism augmented HRP labeling of hypoglossal motoneurons 3 to 4 weeks following axotomy (Yu & Srinivasan, 1981). The thyroxine-provoked increase in neuronal labeling was attributed to polyneuronal reinnervation. The disparity of the results from this and the previous work may reflect distinct HRP injection regimens (Figure 53). In the earlier study, 2 mg of HRP was injected in the midline of the tongue at a single time period. Only neurons innervating the region of the tongue containing HRP would be labeled. However, regenerating neurons, accessing a larger muscle expanse, may be more likely to contact the HRP pool. A larger total amount of HRP (10 mg), injected in a more diffuse manner was utilized in this study, probably effecting greater HRP labeling of uninjured neurons (Olsson et al., 1983). Expansion of the innervation area by a

Figure 53A. Diagram illustrates the HRP labeling procedure utilized in this study. In all eu- and hyperthyroid animals, 10 mg of HRP was injected diffusely into the tongue. This method should maximize HRP distribution in the tongue (indicated by shading) for neuronal uptake. Following regeneration of axons and presumable polyneuronal reinnervation, neuronal labeling with HRP would not exceed that observed among controls.

Figure 53B. This is a diagram of the described HRP injection protocol used by Yu & Srivinasan (1981). Two milligrams of HRP were injected into the midline of the tongue, thus probably resulting in a narrow area of the tongue replete with HRP (shaded area). Following axotomy, axons reinnervating larger muscle area may have greater access to the HRP in the tongue, leading to increased neuronal labeling with the peroxidase.

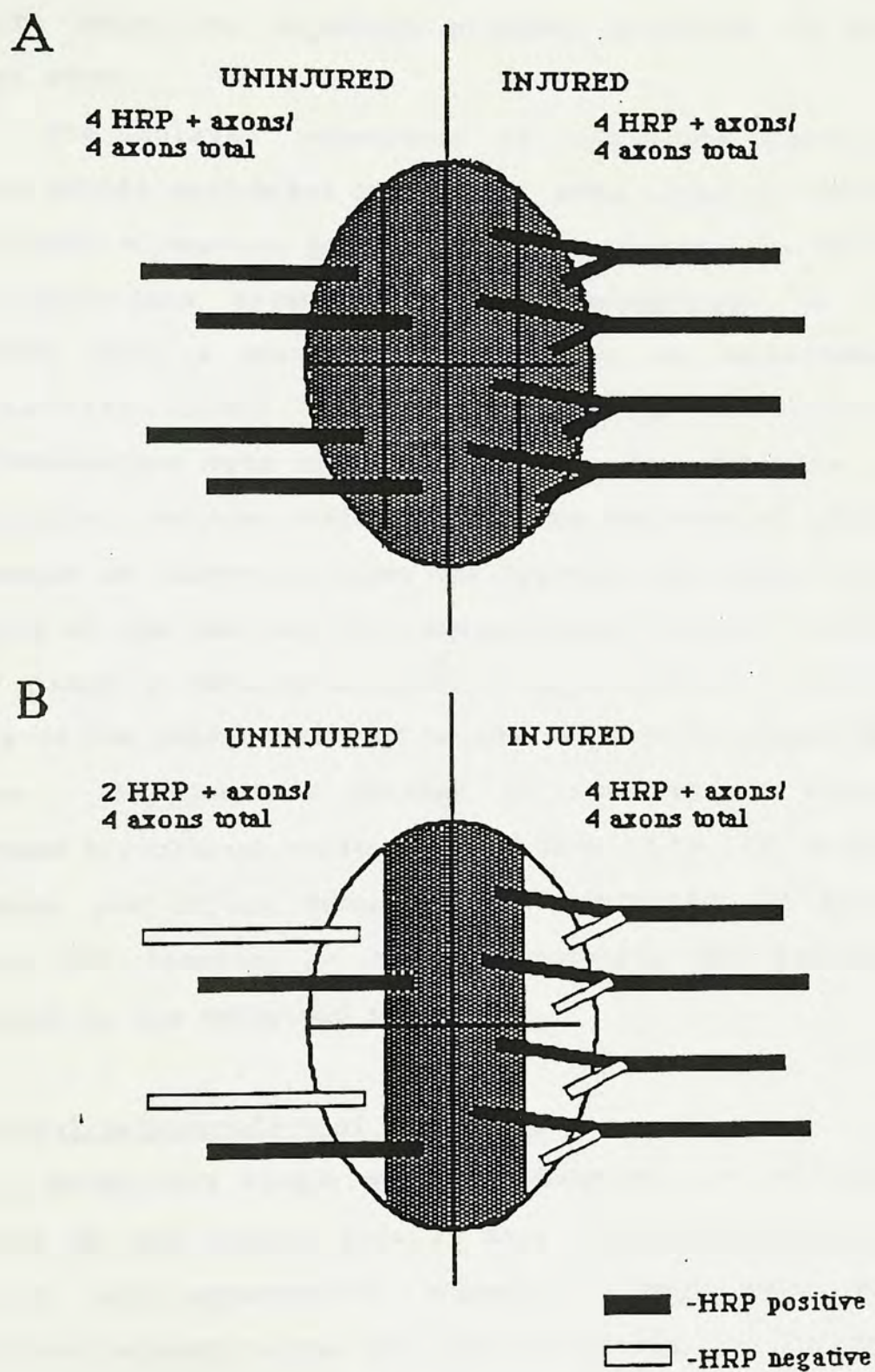


Figure 53

regenerating neuron would probably not serve to increase HRP exposure using the injection protocol described in the current study.

The delayed appearance of peroxidase reaction product within axotomized hypoglossal motoneurons indicated that axonal elongation proceeded at a decreased rate among the hypothyroid animals. This interpretation is in agreement with a previous demonstration of decelerated hypoglossal nerve regeneration among chemically thyroidectomized rats (Talman, 1979). The mechanism by which thyroid hormone deficiency retards the rate of axonal elongation is unknown. Among the hypothyroid animals, HRP labeling of the neuronal cell bodies ranged from 25% of the total number of neurons analyzed 21 days following axotomy to 55% of the total number of neurons 50 days following the lesion. HRP-positive neurons in the injured versus uninjured hypoglossal nucleus ranged from 27 to 79%, during the same post-injury interval. The reduction in post-axotomy HRP labeling of neurons resembles the response exhibited by the euthyroid animals.

Functional Reinnervation of the Tongue

Stimulated tongue muscle contraction was initially detected on the injured side 21 days following axotomy in the eu- and hyperthyroid animals. These data lend functional support to the HRP labeling studies and indicate that recovery of motor function was subsequent to axonal

extension to the tongue. The timing of functional recovery observed among the eu- and hyperthyroid groups is analogous to a previous report (Smith et al., 1984).

In the current investigation, thyroxine treatment failed to hasten the appearance of functional reinnervation. Others have failed to detect these beneficial effects of hyperthyroidism following sciatic and facial nerve injury (Stelmack & Kiernan, 1977; Cotrufo et al., 1979; Allpress & Pollock, 1986). However, analyses of sciatic nerve recovery have indicated that thyroid hormone administration expedites the return of stimulated muscle activity (McIsaac & Kiernan, 1975b) and motor reflexes (Berenberg et al., 1977). The conflicting results are not related to thyroid hormone dose, since similar treatment regimens have elicited both positive and negative results.

Functional reinnervation is the culmination of a complex and multifaceted process including axonal elongation, reinnervation and maturation (Gutmann et al., 1942). Although hyperthyroidism did not detectably accelerate functional recovery in this and other studies, the treatment may have influenced some aspect of the reinnervation process which was not examined. Studies have suggested that particular aspects of regeneration, including synapse formation (McIsaac & Kiernan, 1975a) and myelination (Stelmack & Kiernan, 1977; Allpress & Pollock, 1986), are sensitive to excess circulating thyroid hormone.

Among hypothyroid animals in this study, functional

recovery was delayed beyond 21 days post-axotomy. Thus, despite the failure of excessive thyroxine to enhance the rate of regeneration in this study, an insufficient amount of circulating thyroid hormones decelerated hypoglossal nerve elongation and functional recovery.

Pattern of Force Recovery

In this study, the timing of functional recovery was assessed by measuring tongue force production following stimulation of the nerve. The force recovery trends suggested by this analysis merit brief discussion. The maximal tongue twitch force produced by stimulating the uninjured hypoglossal nerve of control and experimental animals was similar at all periods evaluated, regardless of thyroid hormone status. This observation suggests that hypertrophy of the contralateral, innervated half of the tongue did not occur following unilateral hypoglossal neurotomy.

Among the hypothyroid animals, the return of muscle function was delayed when compared to the euthyroid population. However, the recovery of force production, as reflected by maximal stimulated force, was not impeded by hypothyroidism once the regenerating axons invaded the tongue.

Although the recovery of motor function was temporally parallel among the eu- and hyperthyroid animals, the degree of maximal twitch force produced by the

reinnervated tongue was greater among the hyperthyroid animals than among the euthyroid group. Forty to fifty days following axotomy, among the euthyroid group, the maximal twitch force evoked on the injured versus uninjured side of the tongue averaged 45%. Among the hyperthyroid rats, the percent recovered maximal twitch force averaged 77.5% during the same time period ($p < 0.05$). In a previous study, hyperthyroidism enhanced the recovery of gastrocnemius force production following sciatic nerve crush (Diaz-Guerrero et al., 1947). The increased maximal force production following reinnervation among the hyperthyroid animals was detected whether the nerve or muscle itself was stimulated. This suggests that excessive circulating thyroid hormone may effect not only the regenerating neuron, but, also the denervated muscle target.

THYROID HORMONE STATUS AND THE REGENERATING MOTONEURON: A SUMMARY

The Injured Hypoglossal Motoneuron from Hyperthyroid Animals

Although the rate of axonal regeneration was similar among the eu- and hyperthyroid populations, the prolonged and enhanced neuronal enlargement as well as the dramatic expression of nucleolar vacuolization, observed among the axotomized neurons from the hyperthyroid population, suggest that the thyroxine treatment enhanced neuronal anabolic activity. Only biochemical analysis of this action of thyroid hormone treatment can reveal the specific nature of

the retrograde response of hypoglossal motoneurons from hyperthyroid animals. In this study, diminished labeling of neurons with HRP and accentuated recovery of tongue muscle force production among hyperthyroid animals may indicate some effect of thyroxine on the neuro-muscular axis. Application of in vitro analysis to this system may help specifically identify the muscle versus nerve effects of thyroid hormone.

Hypothyroidism and the Injured Hypoglossal Motoneuron

The structural alterations exhibited by motoneurons from hypothyroid animals resemble some of those seen among immature neuronal populations. Earlier and enhanced cell nuclear changes, characteristic of axotomized hypoglossal motoneurons from the hypothyroid population in this study, have been described among FMN (Soreide, 1981) and hypoglossal motoneurons (Borke, 1979, 1983) in the developing rat. The absence of somal and nucleolar enlargement, also a feature of axotomized motoneurons of the hypothyroid population in this study, has been demonstrated among injured FMN of the immature hamster (LaVelle & LaVelle, 1958).

The shared retrograde responses among injured neurons from hypothyroid and developing animals do not, however, predicate a similar regenerative response. Developing neurons subject to axotomy suffer greater degeneration than corresponding adult populations (LaVelle &

LaVelle, 1958; Borke, 1979; Soreide, 1981). Hypothyroidism did not enhance neuronal cell loss following axotomy through 50 days post-axotomy in this study. The axotomized neurons from young mammals evidence a more rapid reconnection with their targets (Lieberman, 1974). This contrasts with the diminished rate of axonal regeneration observed among regenerating hypoglossal motoneurons. The variable expression of the ultrastructural and regenerative responses to axotomy among developing and hypothyroid animals indicates that axonal regeneration is not related to the cell body response to axotomy in a simple fashion. The elusive significance of structural responses of the neuronal cell body to axotomy may need to be clarified by molecular and biochemical approaches.

Clinical Uses of Thyroid Hormone following Nerve Injury

Many studies of the effect of excessive thyroid hormone on injured nervous tissue have been conducted in hopes of advancing a new clinical approach to patients suffering nerve injury (Harvey & Srebnik, 1967), although only a single, documented clinical study has been attempted (McQuarrie, 1975). Administration of exogenous thyroid hormone following nerve injury, in order to expedite nerve regeneration and recovery, is not indicated by the data from the present study. Although thyroid hormone treatment, in this study, did influence the structure of the injured neuron and, apparently, the functional recovery of the

muscle, the axonal regeneration was not accelerated. In contrast, a sufficient amount of circulating thyroid hormone did prove necessary for the control rate of axonal elongation. Thus, assessment of thyroid hormone status following injury to nervous tissue is recommended. Hypothyroidism should be ameliorated to yield optimal conditions for nerve regeneration.

APPENDIX

Abbreviations

ANOVA - analysis of variance
CNS - central nervous system
DAB - diaminobenzidine
dpa - days post-axotomy
FMN - facial motor neurons
HRP - horseradish peroxidase
NGF - nerve growth factor
RER - rough endoplasmic reticulum
RGC - retinal ganglion cells
S_v - surface density
T₃ - triiodothyronine
V_v - fractional volume
XIIth - hypoglossal

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